EXPERIMENTAL CELL RESEARCH

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LOCALIZATION OF DEOXYRIBONUCLEASE IN TISSUE SECTIONS

A NEW APPROACH TO THE HISTOCHEMISTRY OF ENZYMES

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The Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England

Received September 3, 1956

ETHODS currently used to localize enzymes in tissues consist in dipping sue sections into a solution containing a suitable substrate and precipitating e products of reaction in a visible form. These products must adhere onto e sections with a minimum of diffusion from the sites of enzyme activity , 8].

In the present work, a different approach was investigated. The method is place tissue sections in contact with a film of gelatine containing a substrate nd, after exposure, to stain the film for the remaining substrate. The latter hould be attacked in the regions of the film overlying the areas of tissue ossessing enzyme activity and retained in those parts covering inactive reas. Staining of the exposed film and comparison with the corresponding ssue sections should thus reveal the sites of enzyme activity in the tissue. ilms composed of deoxyribonucleic acid (DNA) dispersed in gelatine were sed here in order to localize the deoxyribonuclease (DNAase) present in ne tissue sections.

MATERIAL AND METHODS

The essential steps of the gelatine–substrate film method are illustrated in Fig. 1. ig. 1*a* shows the materials used in their relative positions immediately before xposing the gelatine–DNA film to tissue sections. Fig. 1*b* represents a cross section f the same elements during exposure. Fig. 1*c* shows the tissue sections and the gela- ine–DNA film after separation and staining. A negative image of the reactive por- ions of the sections is seen in the film.

Preparation of gelatine–DNA films.—A 1:1 mixture of 5 per cent gelatine (commer- ial sheet form) and 0.2 per cent DNA[2] is liquefied by heating in a water-bath. One

[1] This investigation was conducted during the tenure of an Exchange Fellowship under the uspices of the British Empire Cancer Campaign and of the National Cancer Institute of Canada. Permanent address: Montreal Cancer Institute, Notre-Dame Hospital, Montreal, Canada.

[2] Highly polymerized DNA prepared from calf thymus by Mr. E. Johns of this Institute ac- ording to the method of Kay, Simmons and Dounce [6].

drop (ca. 0.05 ml) of the mixture is placed on a glass slide and rapidly spread ov a surface of about 2.5 × 4 cm with the tip of the pipette. The slide is left on a lev surface at room temperature until the gelatine–DNA gel has dried. The film is the fixed overnight in neutral 10 per cent formaldehyde to render the DNA insoluble i water. After fixation, the film is washed in three successive baths of distilled wate 15 minutes each, and allowed to dry.

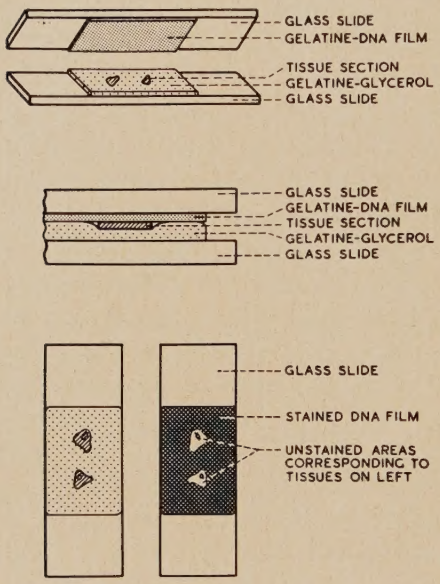


Fig. 1. Localization of DNAase in tissue sections b the gelatine–substrate film method. (*a*, top), m terials used in their relative positions immediate before exposing the gelatine–DNA film to tiss sections. (*b*, center), cross section of the same el ments during exposure. (*c*, bottom), tissue sectio and gelatine–DNA film after separation and stai ing. A negative image of the reactive portion of t sections is seen in the film.

Preparation of tissues.—Male albino rats (ca. 200 g) are killed and the orga excised as rapidly as possible. The organs are placed in a beaker kept on ice, washed i distilled water and blotted on filter paper. A piece of each organ is cut out with scalpel and placed immediately on a layer of ice covering a freezing stage dipped in cracked dry ice. When the tissues are frozen, the stage is adjusted on the freezi microtome kept in an insulated box at ca. −20°C and sufficient time is allowed f the tissues to adjust to this temperature.

Mounting of tissue sections on gelatine–glycerol.—When sections are ready to b cut, a mixture of gelatine and glycerol (ref. 1; final concentrations used: 7 and 40 p cent respectively) is liquefied by heating in a water-bath. About 0.3 ml of th mixture are placed on a glass slide and spread over a surface of approximately 2.5 × cm with the tip of the pipette. The slide is left on a level surface at room temperatu until gelation occurs (5–10 minutes).

Frozen sections are then cut at 15 μ according to the techniques of Linderstrø Lang, Holter and Mogensen (as described in [3]) and the sections are slid with a fi brush from the microtome knife onto the gelatine–glycerol-covered slide. The latt is introduced into the cold box only for the time necessary to transfer the sectio in order to prevent freezing of the semi-solid support. The slide carrying the tiss sections is placed on a level surface at 37°C until the gelatine–glycerol liquefies a

e sections spread on its surface. The slide is then transferred onto a cool level sur-
ce (room temperature) and the gelatine–glycerol support is again allowed to form a
m gel (10–20 minutes). This procedure of allowing the support to liquefy and reset
events the sections from adhering to the gelatine–DNA film when the slides are
parated after exposure.

Exposure of gelatine–DNA films to tissue sections.—The slide carrying the sections
hen placed against the dry gelatine–DNA film and the two are held in close contact
pressure. After standing at room temperature for a given period of time, they
separated again. The gelatine–DNA film is washed in two successive baths of
tilled water, 5 minutes each, and allowed to dry. The slide supporting the tissue
tions is placed in neutral 10 per cent formaldehyde, in a horizontal position, and
t in the fixative overnight. It is then washed in two successive baths of distilled
ter, 5 minutes each, and left aside until the gelatine–glycerol support has dried.

Staining of gelatine–DNA films and tissue sections with toluidine blue.—The dry
latine–DNA film is dipped into a 0.2 per cent solution of toluidine blue for 10 min-
es. The slide is then washed in distilled water to remove excess dye, the film is
owed to dry again and directly mounted with Canada balsam. Control films (2.5
r cent gelatine) do not show any appreciable staining in the same conditions.

The slide carrying the tissue sections is dipped into 1:3 acetic alcohol for 5 minutes
fore staining with toluidine blue. This prevents the staining of the supporting
edium which contains a higher amount of gelatine than the gelatine–DNA film.
he slide is transferred directly from acetic alcohol to a 0.1 per cent solution of
luidine blue. After 1–2 minutes in the latter solution, the slide is washed in distilled
ater to remove excess dye, allowed to dry at room temperature and mounted by
ding a drop of Canada balsam and covering with a coverslip.

RESULTS

Effect of DNAase solution on gelatine–DNA films.—Formaldehyde-fixed
latine–DNA films treated with a solution of DNAase[1] at 37°C showed a
ogressive loss of DNA with time (Fig. 2). While a slight effect was observed
ter 2 hours, treatment for 8 hours resulted in an appreciable decrease in
ainability, and a complete removal of the DNA was observed after 24 hours.
he control solution had no effect on the films in the same conditions.

Effect of tissue sections on gelatine–DNA films.—When exposed to tissue
ctions, the gelatine–DNA films showed a loss of DNA in those regions in
ntact with sections. Fig. 3 illustrates some of the negative patterns left in a
m after exposure to sections of the *small intestine* (Fig. 4) for 1 hour and
aining with toluidine blue. The negative images corresponding very well
the sections are due to the removal of DNA which is attributed to the
drolytic action of the DNAase contained in the tissue sections.

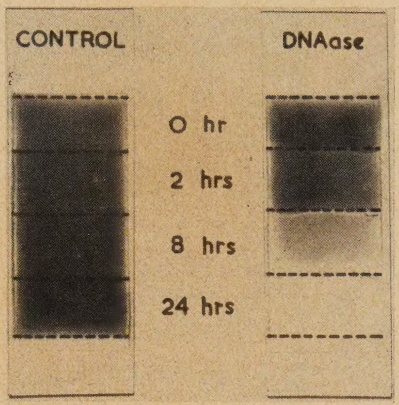
[1] The DNAase solution was prepared by adding 0.1 mg of crystalline DNAase (Worthington
emical Lab.) per ml of McIlvaine's buffer at pH 6.5 containing magnesium sulfate at a final
ncentration of 0.005 *M*. The control solution contained the same reagents but no DNAase.

Examination of sections and corresponding autographs at higher magnification (using a yellow filter) reveals that the DNAase activity is shown mainly by the epithelial cell layer of both the villi and the crypts of Lieberkühn (Figs. 5 and 6). Weaker reactions are given by the lamina propria, the submucosa and the muscle layers but an intense reaction, assigned to a blood vessel, is also observed in the submucosa. The reaction associated with the villi epithelium is discontinuous along the epithelial cell layer and clearly extends beyond the surface into the lumen. The presence of DNAase in the lumen is particularly evident where the spaces between the villi are relatively narrow. These spaces appear filled with the enzyme. The areas of activity associated with the crypts of Lieberkühn do not seem, on the other hand, to cover the entire diameter of the crypts. They rather correspond to the central part of the glands, i.e. the apical portion of the cells and the lumen. Presumably, the DNAase is contained in the excretory region of the goblet cells of both the crypts and the villi epithelia and is discharged into the lumen together with the mucus.

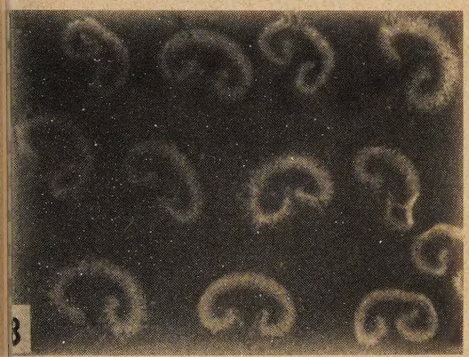
For studying the reaction given by the most active parts of a tissue, autographs of higher resolution can be obtained by reducing the time of exposure. Fig. 7 shows the negative image left in a film after contact with a section of the duodenum (Fig. 8) for 30 minutes, and Fig. 9 shows the results obtained by a 10 minute exposure to a section of the ileum (Fig. 10). These autographs clearly illustrate the presence of DNAase in the spaces between the villi. Mucus secretion can be observed in the same location in the corresponding sections. Moreover, focal reactions which may be assigned to individual goblet cells can be distinguished along the region of the villi epithelium, on each side of the active areas associated with the pits (Fig. 7, left part). These individual reactions are still more evident in some autographs of the crypts of Lieberkühn (Fig. 9, bottom left corner).

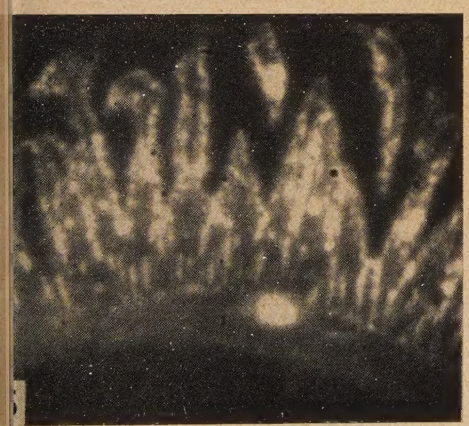
With the *pancreas*, a negative pattern corresponding to the acinar cells was obtained (Figs. 11 and 12). A multifocal reaction was sometimes observed in adjacent acini, suggesting that the DNAase is not evenly distributed in the acini but more concentrated in their centers, i.e. in the secretory pole of the cells.

In the *thyroid*, DNAase activity was shown by the colloid present within the follicles, the intensity of the reaction varying from one follicle to another (Figs. 13 and 14). The follicular epithelium itself was relatively inactive. The interfollicular connective tissue showed a multifocal reaction which may be due to the small blood vessels present in this location.

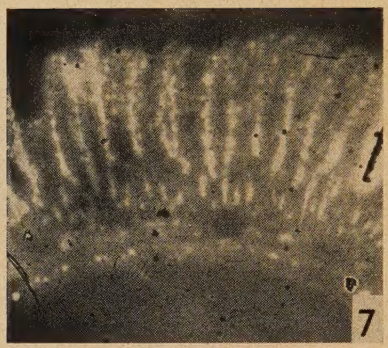


g. 2. Gelatine–DNA films treated with control or NAase solutions for different periods of time and ained with toluidine blue.
g. 3. Gelatine–DNA film exposed to sections of the nall intestine for 1 hour and stained with toluidine ue. Magnification ×2.5.
g. 4. Corresponding tissue sections (×2.5) stained ith toluidine blue.





g. 5. Part of a negative image left in a gelatine–DNA film exposed to sections of the small testine for one hour (Fig. 3) seen at higher magnification (×40).
g. 6. Corresponding tissue section at same magnification.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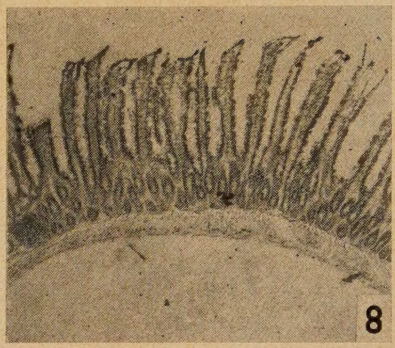
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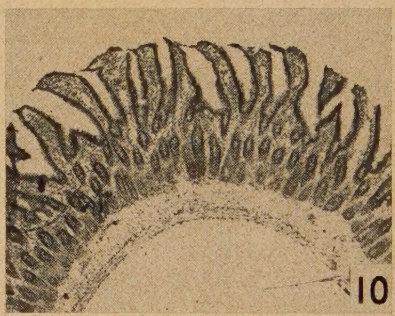
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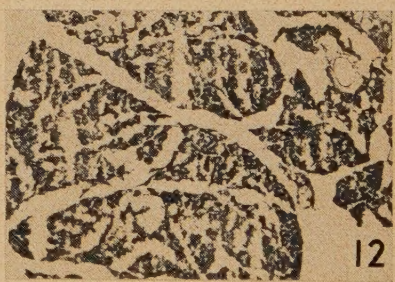
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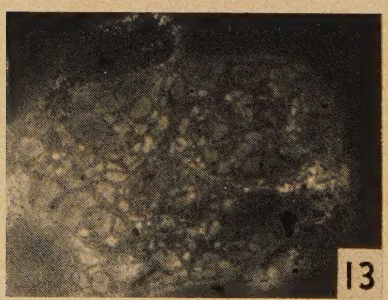
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DISCUSSION

This first attempt to localize enzymes in tissues by the use of gelatine–
bstrate films has proved successful and the new method seems to present
ıny advantages. Firstly, fresh tissue sections can easily be used in this
ethod, thus permitting to localize several enzymes which are inactivated or
ssolved by the treatments necessary in the usual histochemical techniques
. Secondly, the demonstration of enzyme activity is here directly related
changes in the substrate instead of being associated with the formation of
gradation products. This eliminates several problems encountered in the
ual procedures such as suitable reactions for precipitating the end-prod-
ts in a visible form, diffusion of these products from their sites of forma-
n, adsorption on inactive parts of the tissue, etc. [2, 4, 5, 8]. In addition,
e method offers the advantage of being applicable to biological fluids.
nce films of substrate can be used with solutions as well as with tissue
tions, they may also find applications in related fields: studies on enzymes
ecificity, comparisons between cellular and purified enzymes, etc.

A disadvantage of the method is its lower resolution, as compared with
e end-product precipitation methods which can reveal the intracellular dis-
bution of enzymes. A localization at that level could sometimes be achieved
the present method in special cases where the cells present a par-
ular arrangement. The studies on the small intestine, for instance, have
ggested that active DNAase is contained in the secretory region of the goblet
ls and absent from their base. However, the method, in its present state
least, is not generally applicable to the cellular level. The resolution of
e method depends upon several factors: the thickness of the film, the con-
ntration of the substrate in that film, the distance between the sections and
e film, and the duration of the exposure. The composition of the semi-solid

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. 7. Negative image left in a gelatine–DNA film exposed to sections of the duodenum for 30
nutes and stained with toluidine blue. Magnification × 40.
. 8. Corresponding tissue section (× 40) stained with toluidine blue.
. 9. Negative image left in a gelatine–DNA film exposed to sections of the ileum for 10 minutes
l stained with toluidine blue. Magnification × 40.
. 10. Corresponding tissue section (× 40) stained with toluidine blue.
. 11. Negative image left in a gelatine–DNA film exposed to sections of the pancreas for 1 hour
l stained with toluidine blue. Magnification × 40.
. 12. Corresponding tissue section (× 40) stained with toluidine blue.
. 13. Negative image left in a gelatine–DNA film exposed to sections of the thyroid for 5
nutes and stained with toluidine blue. Magnification × 40.
. 14. Corresponding tissue section (× 40) stained with toluidine blue.

support for the sections is also important. It was observed that the addition of glycerol to the support greatly improved the precision of the autographic image, presumably by reducing the rate of enzyme diffusion. The results described above were obtained under the optimum conditions determined after series of assays. While the property of enzymes to diffuse through a thin film is actually the basis of the method, the optimum conditions will naturally be those which will minimize the geometrical affects of enzyme diffusion, a situation in many ways similar to that encountered in radioautography.

The present approach, which has proved practical for localizing DNAase activity, is probably applicable to many different enzymes since suitable films of several substrates can likely be prepared. To visualize the unattacked substrate remaining in the film after exposure, any staining or other method can be used. This procedure does not need to be specific for the substrate, as long as the second component of the film, gelatine, does not stain by the same technique. Thus, the method seems to offer interesting advantages and should prove fruitful in various studies.

In comparing methods for localizing enzyme activities in tissues, no mention was made of the use of fluorescein-labelled antibodies [7]. In that method, the reaction localizes the enzymes as proteins, i.e. does not distinguish between the inactive and the active forms of enzymes, while the methods discussed above localize selectively the active fraction of these proteins. An interesting application of the antigen-antibody reaction might be its use in conjunction with methods based on the activity of enzymes to localize, by difference, the inactive or latent form of enzymes.

SUMMARY

A new approach to the localization of enzymes in tissues was investigated. The method is to place tissue sections in contact with a film of gelatine containing a substrate, to allow the tissue enzyme to act upon its substrate in the film and, after separating the sections and the film, to stain the latter for the remaining unattacked substrate. The latter is attacked in the regions of the film overlying the areas of tissue possessing enzyme activity and retained in those parts covering inactive areas. Comparing the negative pattern left in the film with the corresponding tissue sections reveals the sites of activity in the tissue.

Films made of deoxyribonucleic acid dispersed in gelatine were used in order to localize deoxyribonuclease in tissue sections. The method proved

isfactory for this enzyme which was thus localized in the small intestine, ncreas and thyroid of the rat. The method appears applicable to the local- tion of other enzymes.

wish to thank Prof. A. Haddow for his interest in the present study, Prof. J. A. V. ler, Dr. P. F. Davison and Mr. E. Johns for supplying the preparations of deoxy- nucleic acid, and Mr. K. G. Moreman and his staff for preparing the illustrations. his investigation has been supported by grants to the Chester Beatty Research titute (Institute of Cancer Research: Royal Cancer Hospital) from the British pire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Re- rch, the Anna Fuller Fund and the National Cancer Institute of the National titutes of Health, U.S. Public Health Service.

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METABOLISM OF CHICK EMBRYONIC HEART EXPLANTS DURING TRANSITION FROM *IN VIVO* TO *IN VITRO* CONDITIONS

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Received September 4, 1956

PROBABLY the most remarkable phenomenon observed in tissue cultures is the rapid commencement of growth and cell division shortly after explantation. Under the special conditions of organ culture this tendency may be checked and the cells may be encouraged to differentiate but under the conditions that have most commonly been employed the explant rapidly becomes disorganised and ultimately only one type of primitive cell is to be found. These cells are usually regarded as fibroblasts and appear to be capable of proliferating indefinitely in favourable conditions. This ability is in marked contrast to the situation *in vivo* where such unorganised growth would lead to destruction of the organism. If a mechanism operates to control the proliferation of cells *in vivo* the gradual or sudden loss of such control might be reflected in changes in the pattern of metabolism during the early stages of growth *in vitro* and this study was undertaken in an attempt to detect changes of this kind. Many studies of the metabolism of chick embryonic heart tissue have been made by previous investigators and, although none of these have been conducted in the earliest stages of culture, sufficient information was already available to indicate that it would be the most suitable tissue for preliminary studies.

METHODS

Tissue culture technique.—The tissue was grown directly on glass, without a plasma clot, in a medium consisting of 30 per cent horse serum, 30 per cent chick embryo extract (50 per cent) and 40 per cent Hanks' balanced salt solution. The medium also contained the following antibiotics: penicillin (50 U/ml); streptomycin (50 μg/ml); mycostatin (20 μg/ml). This type of medium was chosen in preference to one of the synthetic media since the behaviour of initial explants in it was well known.

The hearts were removed from 15-day chick embryos and the atria and large vessels dissected off. They were then cut into uniform cubic explants of 0.5–0.6 mm

le by the technique described in a previous communication [14]. The explants re suspended in a quantity of medium equivalent to 1 ml for each vessel to be oculated plus 1 ml for each control specimen plus 2 ml extra. After the explants d settled, the supernatant medium was pipetted off to remove débris. The volume s then restored with an equal amount of fresh medium. A sterile pasteur pipette rather large bore was fitted with a rubber teat pushed well down over the top d so adjusted that compression of the teat delivered exactly 0.5 ml. The explants re stirred with this pipette and when thoroughly and uniformly suspended 0.5 ml material was snatched into it by releasing the compressed teat. This was dispensed a culture vessel and the procedure repeated along the line. When all vessels had eived 0.5 ml in this way the process was repeated but working backwards from e last vessel inoculated to the first. Initial (control) samples of tissue were inoculated o centrifuge tubes distributed among the other vessels. By this means each vessel eived 1 ml of inoculum and the variation was found to be well within 10 per cent. edium was then added to each vessel to make the required volume, after which ey were placed in the incubator and left strictly undisturbed. The explants adhered the floor of the vessel after one or two days and cells migrated over the glass. stological examination showed that the explants, whether adherent to the glass not, formed a capsule of fibroblasts while necrosis occurred to a greater or lesser tent in the centre after a few days.

Collection and analysis of specimens. —The explants were dislodged from the floor the vessel by a glass scraper and removed by a wide-mouthed pasteur pipette ng with the medium, great care being taken that all the tissue was collected. ey were placed in a centrifuge tube and the tissue was separated from the medium centrifugation and decantation. The tissue was subsequently analysed by methods scribed by Paul [19]. The medium was deproteinised and analysed for carbohydrates d pyruvic acid as described by Leslie and Paul [14]. Lactate was also estimated the medium by the method of Hullin and Noble [9].

Measurement of respiration.—As described below, some cultures were grown in rile Warburg flasks and oxygen uptake was determined in the usual manner as quired. It is particularly necessary to grease the lip of the inner cup in order to event creeping of alkali since the glass is always likely to be moist after incubation r several days.

Measurement of ^{32}P incorporation.—This was carried out simultaneously with spiration. 20 μc in 0.2 ml balanced salt solution were placed in the side-arm of e Warburg flask and added to the contents of the main vessel exactly two hours fore the termination of the experiment. The tissue was fractionated by a modified hmidt and Thannhauser procedure [3] and the specific activity of the phosphorus the different fractions determined in the usual manner.

Design of experiments.—Two types of conditions were employed. In the first oup of experiments a high ratio of medium to tissue was maintained. 15–30 mg sh weight of explants were grown in modified Kolle flasks with 15 ml of medium. he medium was not renewed and samples were withdrawn for analysis at intervals to six days after explantation. In the second group of experiments a low ratio medium to tissue was maintained. Approximately the same amounts of tissue re grown in 2 ml of medium in Warburg flasks, the medium being changed com-etely at 48 hours and, in experiments running beyond this, every 48 hours there-

after. Respiration was measured periodically and in some experiments ^{32}P incorporation also. Alkali and a filter-paper tube were added to the centre cups immediately before measuring respiration and tissue and medium were harvested immediately after. The ratio of medium to tissue in these latter experiments approximates to that in the classical coverslip culture with two rather large explants in one drop of medium.

RESULTS

Changes taking place in the chemical composition and histological structure of chick embryonic heart and liver during the transition from *in vivo* to *in vitro* conditions form the subject of a separate investigation [20]. In this study reference will therefore be made only to those findings which are directly relevant to the observations under discussion.

Table I shows typical findings for the deoxyribonucleic acid (DNA) and carbohydrate content of chick embryonic heart tissue cultured in flasks according to the conditions described. With regard to DNA the outstanding facts are that the initial fall is rather less than that reported previously while on the other hand it is considerably prolonged [2, 3, 6]. The significance of this is suggested by histological studies which show that, while there is good survival of all cell-types initially, the muscle cells at the centre of the explants later tend to die, being replaced by fibroblasts at the periphery. There is no question of these cultures not being properly designated "surviving" or "growing" cultures because pulsation of fragments has been observed up to 14 days after explantation and strains surviving for many weeks have been produced from them. Also histology reveals that there are always a large number of healthy fibroblasts present and that, although many muscle cells die, others survive for the duration of the experiment at least [20].

The carbohydrate (hexose) content of the explants is seen to decrease

TABLE I

Changes in deoxyribonucleic acid and carbohydrate content of 15-day chick embryonic heart explants during the first 96 hours *in vitro*.

	Time in hours				
	0	5	24	48	96
Deoxyribonucleic acid, μg	4.01	3.18	3.11	2.57	2.52
	± 0.36	± 0.34	± 0.78	± 0.09	± 0.15
Hexose, μg	207.1	109.2	119.8	91.9	75.8
	± 40.9	± 11.14	± 11.4	± 18.3	± 9.3

TABLE II

nges in the composition of the medium during the first six days of growth of chick embryonic heart explants *in vitro*.

igh ratio of medium to tissue was employed and the medium was not changed. The culture vessel was not agitated. Samples of medium were analysed at the times shown.

		Hours of incubation						
pt.	Obs.	0	5	24	48	72	96	144
rose								
/ml								
C1	1	1.354	—	0.664	0.500	0.348	0.188	0.136
C2	1	1.576	—	1.585	1.508	1.419	1.124	1.040
C4–9	6	1.575	1.518	1.217	0.904	0.776	0.672	0.583
			±0.092	±0.108	±0.046	±0.071	±0.061	±0.053
tate								
/ml								
C1	1	0.197	—	0.571	0.614	0.532	0.554	0.562
C2	1	0.197	—	0.316	0.336	0.495	0.696	0.882
C4–9	6	0.440	0.533	0.752	0.917	1.030	1.040	0.918
			±0.028	±0.063	±0.097	±0.073	±0.057	±0.124
uvate								
ml								
C1	1	3.44	—	33.9	36.8	41.8	31.9	24.1
C2	1	3.00	—	15.7	31.4	33.2	28.6	36
C4–9	6	9.60	13.5	36	48.1	50	49.2	38.5
			±1.37	±2.67	±4.04	±2.55	±2.79	±2.18

idly, a finding which agrees with earlier histochemical findings [21]. the conditions employed, about 100 μg of endogenous hexose may be ntributed to the system within the first few hours *in vitro*.

Table II summarises the glucose, lactate and pyruvate findings in experi-nts in which the tissue was grown in an excess of medium which was not ewed for six days. Ample verification was obtained of the observations previous investigators that chick embryonic heart tissue readily utilises ucose with the production of lactate [4, 11, 12, 15, 16, 18, 23, 24]. It is o clear that, as shown previously [14], pyruvate accumulates in the dium in the presence of metabolising tissue. It is interesting to observe t in the late stages of some of the experiments, where the glucose level d fallen very low, there was definite evidence for utilisation both of tate and pyruvate, as had been suggested by previous workers [24]. A

fall in the level of pyruvate in the medium tended to precede the fall in lactate where this occurred.

Fig. 1 shows an analysis of experiments 1C4 to 1C9, arranged to demonstrate the balance between the rate of glucose utilisation and the rate of formation of its products. It immediately becomes obvious that the amount of glucose utilised within the first few hours does not account for the amount

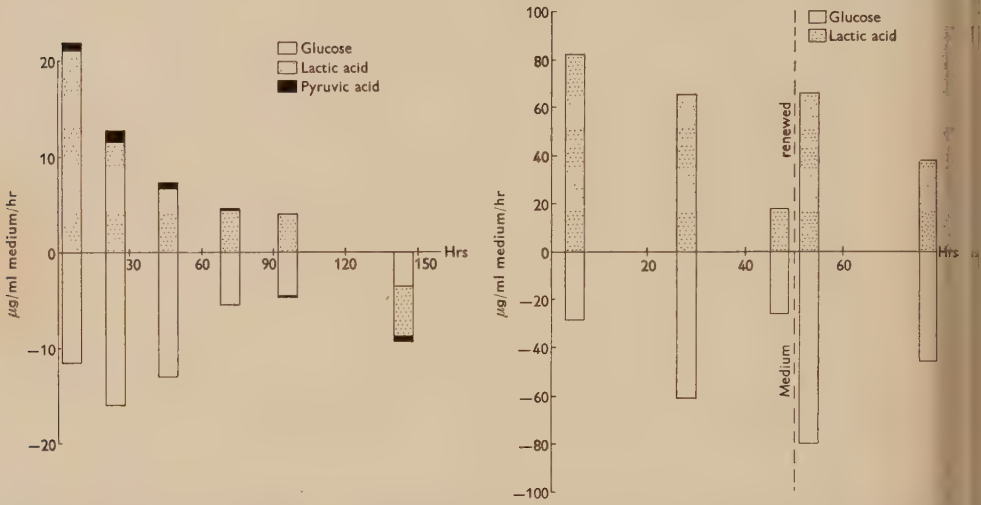


Fig. 1 (left). Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic heart tissue. The amount of each substance utilised or produced per hour was calculated from the change in concentration since the previous observation. A high ratio of medium to tissue was employed and the medium was not renewed.
Fig. 2 (right). Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic heart tissue. A low ratio of medium to tissue was employed and the medium was completely renewed after 48 hours *in vitro*. The amount of each substance utilised or produced was calculated as in Fig. 1.

of lactate and pyruvate produced. Willmer [23] observed a similar phenomenon. A part of this may be accounted for by the release of endogenous carbohydrate (Table I) and its utilisation but not by any means all of the lactate can be accounted for in this way. It may arise from deamination of amino acids and utilisation of the residues [5] or merely from other intermediary metabolites present in the medium. After 24 hours the rate of glucose utilisation has increased while the rate of lactate production has fallen and now lactate accounts for only about 70 per cent of the glucose used. Subsequently both the rate of glucose utilisation and the rate of lactate production decrease until, towards the end of the experiment, there is utilisation of glucose, lactate and pyruvate.

TABLE III

anges in the composition of the medium during the first three days of growth of chick embryonic heart explants *in vitro*.

ow ratio of medium to tissue was employed and the medium was renewed after 48 hrs. The ture vessel was agitated to permit respiratory measurements during the last 4 hours of each period. Otherwise the vessel remained stationary.

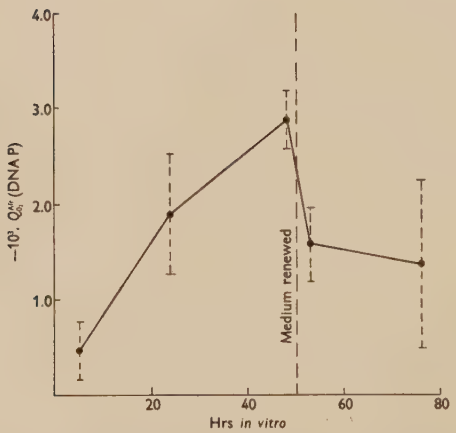
pt.	Obs.	0	5	28	48		53	79
rose								
/2ml								
G1	3	2.67	2.505 ±0.2	0.908 ±0.322	0.430 ±0.06		2.16 ±0.068	0.965 ±0.128
G2	3	2.558	2.455 ±0.14	1.331 ±0.353	0.758 ±0.07		2.337 ±0.051	1.231 ±0.055
G3	3	2.758	2.587 ±0.11	1.191 ±0.033	0.697 ±0.17		2.307 ±0.12	1.308 ±0.004
tate								
/2ml								
G1	3	1.156	1.062 ±0.166	3.078 ±0.459	5.165 ±0.243	medium removed	1.521 ±0.047	2.165 ±0.219
G2	3	0.820	1.228 ±0.24	2.471 ±0.09	2.807 ±0.232		1.229 ±0.062	2.227 ±0.084
G3	3	0.938	1.576 ±0.105	2.800 ±0.3	3.435 ±0.172		1.049 ±0.014	2.085 ±0.05
ruvate								
2ml								
G1	3	14.4	80.9 ±18.12	98.9 ±10.72	128 ±10.55		36.6 ±0.082	73 ±10.31
G2	3	15.43	67.4 ±5.17	100.3 ±11.3	105.2 ±11.95		26.24 ±4.7	78.7 ±12.64
G3	3	13.32	70.9 ±4.5	91.97 ±10	89 ±17.3		35.3 ±1.3	68*

* Single observation.

In Table III are summarised the findings in experiments in which the sue was grown in a limited amount of medium which was renewed after hours. Fig. 2 presents an analysis similar to the above. It is apparent t the pattern of metabolism in the first 48 hours is similar to that found in previous experiments but that the changes occur much more rapidly d are more extreme. On renewal of the medium there is no tendency to

revert to the picture obtained in the first five hours of culture. Instead there is a high rate of glucose utilisation accompanied by production of lactate and pyruvate which together account for rather more than 80 per cent of the glucose. In the next 24 hours utilisation of glucose and production of lactate decrease.

These observations would suggest that the utilisation of lactate and pyruvate in the late stages of the experiments illustrated in Fig. 1 were due



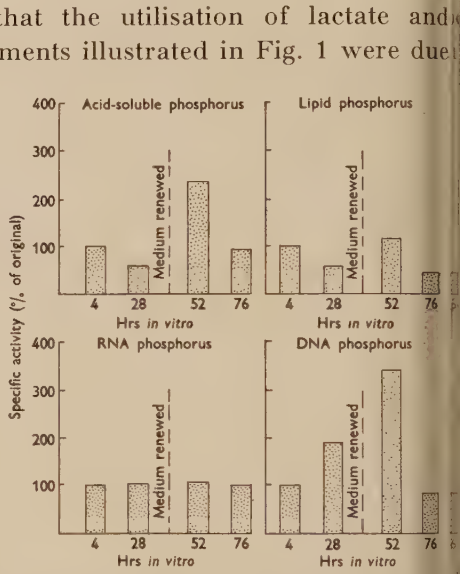


Fig. 3 (left). The respiration of chick embryonic heart tissue during the early stages of growth *in vitro*. The conditions of culture were the same as in Fig. 2. Means and standard deviations of at least nine observations are shown in each case.

Fig. 4 (right). The incorporation of ^{32}P (as inorganic phosphate) into fractions of chick embryonic heart tissue. The conditions were the same as in Fig. 2. Incorporation was allowed to proceed for two hours exactly. The columns express specific activity as a percentage of the initial specific activity in each fraction. Initially the specific activities in relation to DNA (= 1) were as follows: ASP—13.9, LP—1, RNAP—2.44.

rather to the exhaustion of available glucose from the medium than to fundamental changes in tissue metabolism.

The respiration of the fragments was measured in similar conditions. As shown in Fig. 3 a very distinctive pattern was obtained. Immediately after removal from the embryo the respiration of chick heart tissue in growth-promoting medium was found to be very low indeed. During the next 48 hours it increased progressively and on renewal of the medium there was an immediate, statistically significant, fall. Respiration never fell as low as the original level, however. The explanation of this behaviour is obscure but it seems likely that it is related to the utilisation of lactate when the

cose level in the medium has fallen low. While this pattern of respiration very reproducible in the conditions described, we have occasionally served that slight changes in the conditions may give rise to enormous ferences in respiration, a $Q_{O_2}^{Air}$ (DNAP) of -25×10^3 having been observed imes. Some of the factors involved are obscure and are being investigated. wever, even when such phenomena did occur the initial respiration was ays lower than that found subsequently.

The uptake of ^{32}P by different fractions of tissue cultures has been in-tigated by Hull and Kirk [7] but their experiments present some difficulties interpretation since incorporation was allowed to proceed from the ginning of the experiment to the time at which the cells were harvested. the experiments, illustrated in Fig. 4, incorporation was measured over xed period of time at suitable intervals and thus a more easily interpreted ture of the phosphorus metabolism of the tissue was obtained. Activity the acid-soluble and lipid fractions fell off after 24 hours in unchanged dium, suggesting a general lowering of metabolic activity at this time. On ewing the medium there was a great increase in the specific activity of fractions, rising to levels considerably in excess of those originally found. er a further 24 hours in unchanged medium the activity fell off once re. In the DNA fraction the specific activity increased within the first hours and again increased after feeding but fell off when the medium s not changed later. The activity of the RNA fraction remained at much same level throughout, but it is doubtful whether much significance can attached to any of the findings in this fraction since it is liable to contain ny compounds other than ribonucleic acid, some of which may have a siderably higher activity than it. Owing to the very small amounts of terial available, further purification was not practicable.

DISCUSSION

t has been known for some years that tissues may grow for a time in mpletely, or almost completely, anaerobic conditions [13, 17, 18] provided cose is present in the medium. This suggested that anaerobic glycolysis ght be a particularly important energy-producing pathway *in vitro* and ny experimental observations have tended to confirm this [11, 12, 15, 16, 23, 24]. There is, nevertheless, incontrovertible evidence that tissue cul-es do respire and presumably use oxidative pathways to some extent 11, 12, 18, 22, 25]. In general, previous workers have failed to agree on

estimates of Q_{O_2} and at least part of the explanation is suggested by the present findings, where, in the course of a few days, a considerable fluctuation in values for Q_{O_2} was found. Heart tissue is usually regarded as highly aerobic and it was therefore surprising to find that the initial rate of respiration in growth-promoting medium was very low, although Warburg and Kubowitz had shown, many years ago, that very young embryonic chick heart could be maintained under conditions of strict anaerobiosis [22].

The subsequent increase in respiration is probably partly explained by the tissue oxidising lactate and pyruvate when glucose has been partially exhausted from the medium, although the mechanism for such a switch must remain an intriguing problem. However, this cannot be the whole explanation because after renewing the medium there was never a complete return to the very low Q_{O_2} which existed originally. Not only does the Q_{O_2} remain higher but in most cases the total oxygen consumption of the tissue remains above that found initially, so that it is unlikely that the Q_{O_2} is boosted by respiring cell fragments. Another possible explanation of the phenomenon is that more actively respiring cells may replace those originally present, but the usual histological changes within 48 hours provide little evidence for this. The other possibility is that a change takes place in the metabolism of the tissue within 24 hours of explantation. There is some evidence for such a change in that the original picture of metabolism in the fresh tissue differs in many respects from the picture obtained on renewing the medium 48 hours later. It has been observed that there is always a considerable increase in the metabolic activity of tissues after adding embryo extract [8, 10] but the significant fact demonstrated here is that the response is apparently much greater after 48 hours *in vitro* than it is in fresh tissue. This particular difference cannot readily be attributed to changes in the medium since the same medium is used at 48 hours as at 0 hours and storage in the refrigerator might be expected to lead to some deterioration in its activity rather than to an increase.

There would thus seem to be good evidence for the existence of an initial "lag phase" in metabolism before the tissue adapts itself to *in vitro* conditions. This phase of changing metabolism may be related to the diminution of nucleic acids observed in the first day of explantation. In the light of our present knowledge it is impossible to arrive at any satisfactory explanation of the phenomenon but the following possibilities may be considered: (1) During preparation the tissue may be damaged and it may not respond completely to stimuli until it has recovered. (2) The death of some cells may produce inhibitory metabolites in the earliest stages. (3) Fibroblasts may be

ore responsive to tissue culture conditions than the other cells and may rm a higher proportion of the explant after 48 hours. (4) Some inhibitory echanism existing *in vivo* may cease to function after cultivation for a ort time *in vitro*. (5) Death of some cells may create an imbalance which rmits other cells to grow more vigorously.

While it is impossible to adduce convincing evidence from the information ailable certain comments may be made. In the first place the death of lls observed on histological examination occurs rather at the centre of e explants than at the periphery, which argues against direct injury being important factor. Secondly, if an inhibitory mechanism exists *in vivo* e factors involved are not found in embryo extract. Finally, when cells ve started to proliferate *in vitro* they will continue to multiply so long as an lequate medium is supplied to them. It seems rather unlikely that this is ie to substances released by involutionary processes within the first hour two of cultivation and argues rather in favour of release from some limiting echanism which exists *in vivo*.

SUMMARY

1. 15-day chick embryonic heart explants were grown for up to six days *vitro*. Metabolic measurements were made immediately after explantation id at intervals thereafter.

2. Initially more lactate was produced than could be accounted for by rbohydrate available in the medium and tissue. 24 hours later the lactate oduced accounted for only about 70 per cent of the glucose utilised. Later ill, when the medium was partially depleted of glucose, lactate and pyruvate ere utilised by the tissue.

3. A very low oxygen consumption was found initially. This increased a considerable factor over the next 48 hours. On renewal of the medium spiration decreased significantly but never fell to the original low level.

4. The specific activity of acid-soluble phosphorus and lipid phosphorus creased during 24 hours after explantation when the medium was not newed. On renewal of the medium at 48 hours both activities rose to levels nsiderably in excess of those found in newly explanted tissue. Little change as noted in the specific activity of the ribonucleic acid fraction. The specific tivity of the deoxyribonucleic acid fraction increased after explantation id increased further after renewing the medium. Later, when the medium as not renewed, it declined.

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METABOLISM OF CHICK EMBRYONIC LIVER EXPLANTS DURING TRANSITION FROM *IN VIVO* TO *IN VITRO* CONDITIONS

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the previous study [9] some quantitative differences were demonstrated tween the metabolism of chick heart tissue cultures immediately after plantation and at later stages in growth. It was suggested that these might rtly be due to the change in environment and release from the influence factors operating *in vivo*. It was considered desirable to make similar idies with another tissue, preferably one with a very different pattern of etabolism *in vivo* and liver seemed most suitable from this point of view. has been used widely for tissue culture studies and Chen [2] has demonrated the survival of liver fragments in conditions of nutrition similar to ose we employed for our studies with heart explants. For these reasons ick embryonic liver was chosen as experimental material. Some previous idies have been made of the metabolism of liver cultures [4, 6, 8, 11, 12] it these have not thrown much light on the pattern of metabolism of this sue *in vitro*.

METHODS

Essentially these were identical with the methods described in the previous paper]. Livers from 15-day chick embryos were prepared by removing the gall-bladders d adherent loops of intestine. They were then chopped and planted as described the preceding communication.

The analytical methods employed were exactly the same as those described preously except that, in addition, reducing sugar was estimated by a modification the method of Folin and Wu [5].

Most of these experiments were performed either simultaneously with those on art tissue or alternating with them so that conditions were as nearly identical as ssible. Rather more liver tissue was used than heart tissue because more necrosis ually occurred.

The experiments were designed in exactly the same manner as in the heart studies.

RESULTS

The chemical and histological changes in freshly explanted liver tissue form part of a separate study [10] and only the figures for deoxyribonucleic acid (DNA) and carbohydrate are reported here (Table I).

It was shown many years ago that the nucleic acid content of tissue slices decreased during the first few hours [1] and therefore the same fall in DNA might be expected in liver tissue as has been found in other tissues on explantation [3, 7]. It can be seen that this did, in fact, happen. The fall was both greater and more prolonged than in embryonic heart. Histology nevertheless showed good survival of all tissue elements, though with marked necrosis, particularly of hepatocytes, in the middle of many explants. After three or four days a capsule of fibroblasts had formed and fibroblasts had also grown over the glass floor of the culture vessel.

Previous observers had shown by histochemical methods that some glycogen was lost from liver explants [4, 6, 8] but that the cells still retained an ability to build glycogen and the decrease was not rapid.

As can be seen from Table I, the carbohydrate (hexose) content of embryonic chick liver is very low and even lower in relation to the amount of DNA than in chick embryonic heart. Also, although a fall in the carbohydrate content of the explants was found, it occurred more slowly and to a smaller degree than in heart. The rate of decrease varied to some extent in different experiments, the fall sometimes occurring within 24 hours and sometimes being delayed for two or three days, but in either event only a small amount of carbohydrate became available for utilisation by the tissue.

Roffo [11] described a high rate of glycolysis in liver tissue but his observations were made after some time *in vitro*. In Table II are shown the results of experiments in which the tissue was maintained in an excess of medium,

TABLE I

Changes in deoxyribonucleic acid and carbohydrate content of 15-day chick embryonic liver explants during the first 96 hours *in vitro*.

	Time in hours				
	0	5	24	48	96
Deoxyribonucleic acid, μg	10.45	7.32	4.52	3.54	2.75
	±0.83	±0.67	±0.40	±0.45	±0.27
Hexose, μg	77.2	80.4	72.9	81.2	59.2
	±10.3	±22.9	±8.8	±10.7	±5.8

TABLE II

anges in the composition of the medium during the first six days of growth of chick embryonic liver explants *in vitro*.

igh ratio of medium to tissue was employed and the medium was not changed. The culture vessel was not agitated. Samples of medium were analysed at the times shown.

		Hours of incubation						
pt.	Obs.	0	5	24	48	72	96	144
xose								
/ml								
C1	1	1.354	—	0.832	0.664	0.248	0.072	0.082
C2	1	1.576	—	1.524	1.484	0.990	0.708	0.560
C4–9	6	1.451	1.576	1.524	1.199	0.958	0.770	0.633
			±0.046	±0.071	±0.077	±0.032	±0.082	±0.017
tate								
/ml								
C1	1	0.197	—	0.406	0.508	0.440	0.489	0.354
C2	1	0.197	—	0.089	0.224	0.643	0.866	0.954
C4–9	6	0.525	0.409	0.360	0.652	0.779	0.837	0.823
			±0.021	±0.056	±0.069	±0.052	±0.039	±0.078
ruvate								
ml								
C1	1	3.44	—	28.0	32.9	22.2	16.2	17.2
C2	1	3.00	—	8.52	18.42	21.9	15.7	16.6
C4–9	6	9.6	10.6	22.1	20.9	19.4	18.4	13.9
			±1.9	±1.9	±2.15	±2.26	±1.2	±4.25

ich was not renewed. The most striking feature is the evidence for utilisa-n of lactate in the first few hours accompanied by an increase of hexose the medium. Simultaneously, it is interesting to note that there is a produc-n of pyruvate by the tissue.

In Fig. 1 the results of experiments 1C4 to 1C9 have been analysed to ow the balance between glucose and its products in the medium. This alysis makes it clear that not all of the hexose and pyruvate produced is counted for by lactate utilised. The difference is not large, however, and uld be accounted for satisfactorily by the release of endogenous carbo-drate. By 24 hours the picture has changed considerably. Although lactate still being utilised during this period there is also evidence for utilisation hexose. Later still the metabolic picture has become completely reversed, xose and pyruvate being utilised with the production of lactate. Over per cent of the hexose is accounted for as lactate. In subsequent days both

glucose utilisation and lactate production diminish, the latter more rapidly than the former, until, after six days have elapsed, there is very little lactate production at all.

When the tissue was grown in a limited amount of medium, renewed after 48 hours, the values for hexose, lactate and pyruvate were as shown in

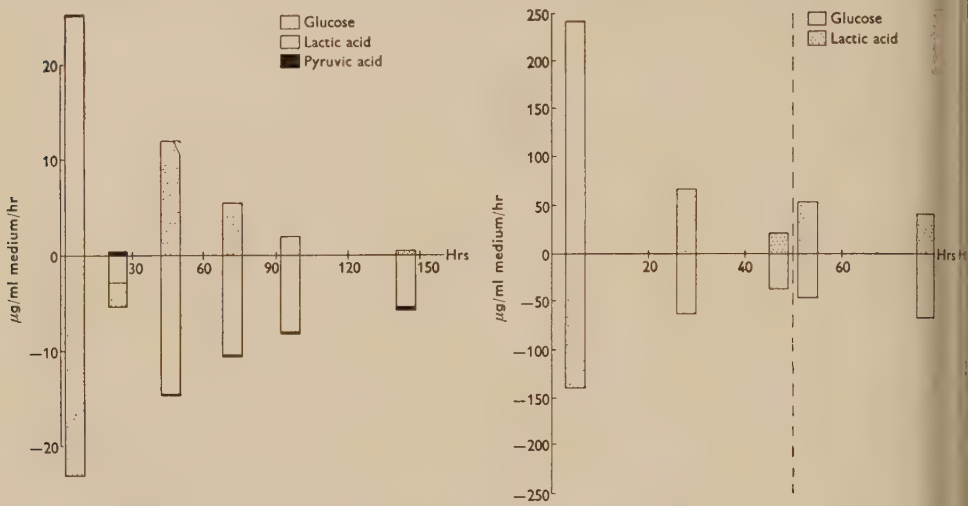


Fig. 1 (left). Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic liver tissue. The amount of each substance utilised or produced per hour was calculated from the change in concentration since the previous observation. A high ratio of medium to tissue was employed and the medium was not renewed.
Fig. 2 (right). Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic liver tissue. A low ratio of medium to tissue was employed and the medium was completely renewed after 48 hours *in vitro*. The amount of each substance utilised or produced was calculated as in Fig. 1.

Table III. These results have been analysed in the same way as before and are summarised in Fig. 2. Again it is seen that there is considerable utilisation of lactate and production of hexose in the first five hours, the changes being much more marked than in the experiments reported in Table II. Again more hexose is produced than can be accounted for by lactate utilised and on this occasion it is not possible to account for the extra hexose production by release of endogenous carbohydrate. On renewing the medium after 48 hours there is no reversion to the original picture of metabolism. Instead there is a very rapid utilisation of hexose with the production of both lactate and pyruvate, so that the pattern of metabolism at this stage is identical with that observed in chick embryonic heart explants at the same stage. This cannot be accounted for entirely by changes in the cell types in the explants

TABLE III

anges in the composition of the medium during the first three days of growth of chick embryonic liver explants *in vitro*.

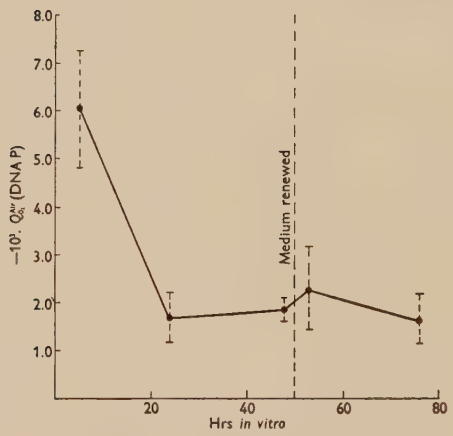
low ratio of medium to tissue was employed and the medium was renewed after 48 hours. ie culture vessel was agitated to permit respiratory measurements during the last 4 hours of each period. Otherwise the vessel remained stationary.

xpt.	Obs.	0	5	28	48	53	79
exose							
g/2ml							
1G1	3	2.67	3.24	2.205	1.462	2.345	1.095
			±0.4	±0.282	±0.177	±0.145	±0.130
1G2	3	2.588	4.47	2.928	2.503	2.445	1.373
			±0.524	±0.344	±0.206	±0.099	±0.108
1G3	3	2.758	3.96	2.276	1.583	2.508	1.144
			±0.242	±0.016	±0.13	±0.095	±0.004
actate							
g/2ml							
1G1	3	1.156	0.507	1.757	2.478	1.352	2.313
			±0.034	±0.479	±0.126	±0.13	±0.019
1G2	3	0.820	0.156	1.999	1.96	1.251	1.923
			±0.005	±0.216	±0.281	±0.222	±0.099
1G3	3	0.938	0.112	1.684	2.39	1.142	2.034
			±0.069	±0.116	±0.05	±0.41	±0.166
yruvate							
g/2ml							
1G1	3	14.4	70.6	65.0	68.8	54.5	73.5
			±4.08	±9.499	±1.79	±4.47	±10.79
1G2	3	15.43	20.33	61.7*	68.3	31.25	55.56
			±3.107		±21.86	±2.70	±7.08
1G3	3	13.32	32.56	45.7	56.3	43.4	53.7
			±7.0	±0.5	±2.04	±5.4	±2.2

* Single observation.

ecause, although there was sometimes considerable central necrosis in ie explants on histological examination, there were nevertheless large umbers of apparently healthy surviving hepatocytes in the samples examined. Thus there is definite evidence for the production of carbohydrate t the expense of lactate in the early stage of explantation and a subsequent eversal of this course of events with continued cultivation. The nature of ie carbohydrate material synthesised is not completely certain but we have stablished the fact that the increase in hexose (determined by the anthrone

method) is parallelled by an increase in reducing sugar (determined by the method of Folin and Wu). Glucose would seem to be the most likely reducing hexose produced by liver tissue in these circumstances. A discrepancy was constantly observed between the amount of hexose and the amount of reducing sugar present in the medium when both were expressed



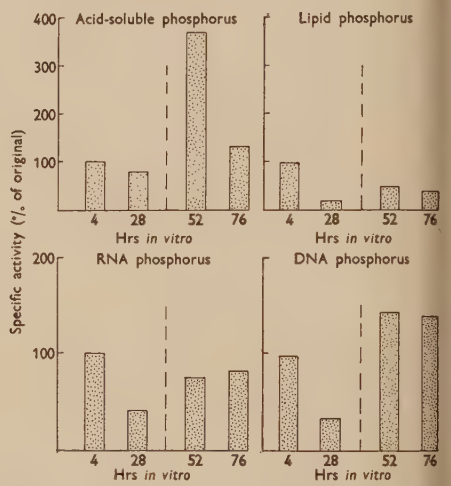


Fig. 3 (left). The respiration of chick embryonic liver tissue during the early stages of growth *in vitro*. The conditions were the same as in Fig. 2. Means and standard deviations of at least nine observations are shown in each case.

Fig. 4 (right). The incorporation of ^{32}P (as inorganic phosphate) into fractions of chick embryonic liver tissue. The conditions were the same as in Fig. 2. Incorporation was allowed to proceed for two hours exactly. The columns express specific activity as a percentage of the initial specific activity in each fraction. Initially the specific activities in relation to DNA (= 1) were as follows: ASP—12.9, LP—1.59, RNAP—2.6.

as glucose. This is apparently due to the existence in the culture medium of some non-reducing hexose.

The respiration of explants growing in Warburg flasks with a limited amount of medium was studied, in many experiments simultaneously with the observations reported in Table II. The initial rate of respiration on removal from the embryo was high and it declined during the next 48 hours. When the medium was renewed after 48 hours no significant effect on the uptake of oxygen was observed. These findings were again in direct contrast to the findings with chick embryonic heart. It has been mentioned in connection with the heart studies that one or two observations were made in which respiration did not follow the typical pattern. This was true also of liver tissue in experiments conducted under the same conditions. Thus the par-

ular pattern described here may be characteristic only of the precise con-ions employed.

In some respects the incorporation of ^{32}P into the phosphorus-containing ctions of liver tissue (Fig. 4) differed from the findings in similar frac-ns of heart tissue. In the acid-soluble fraction the picture was essentially same, a great increase in incorporation following renewal of the medium er 48 hours' growth *in vitro*. On the other hand, while incorporation into id phosphorus was stimulated by renewal of the medium, it never rose the initial level, a situation which contrasts with heart tissue. Incorporation o DNA decreased during the first 24 hours but was considerably stimulated renewal of the medium. As in the case of heart tissue, it is dubious whether y valid conclusions can be drawn from the results obtained for the RNA action.

DISCUSSION

Changes in the medium during cultivation of chick embryonic liver have en studied previously by only two groups of workers. In one case liver is mixed with other tissues [13] while in the other, observations were gun only after the explants had been in culture for some time [11]. On lowing the changes in metabolism from the earliest stage of explantation, has been shown here that a complete reversal occurs within 24 hours in sue culture. The reasons for this are of considerable interest and several planations appeared possible. In the first place it seemed that the death liver cells and their replacement by fibroblasts might provide an adequate planation but, as has been mentioned, the histological picture of explants er 24 hours' cultivation does not provide convincing evidence for this. n the other hand it was possible that the exhaustion of lactate from the edium resulted in the appropriate metabolic pathways ceasing to function operly, or that an inadequate supply of oxygen might produce the same ect. Some unreported experiments were designed to investigate these ssibilities and it was found that addition of lactate to the medium and ltivation of the tissue in roller-tubes did not prevent the change from taking ace. It thus seems likely that the withdrawal of normal environmental fluences plays a vital part in the switch of metabolism and that these fluences are not restored by embryo extract.

It is interesting to observe that the metabolic pattern which rapidly evolves these conditions is very similar to that which develops in chick embryonic art cells. While, particularly in the later stages, this may be due to over-owth of the other tissue elements by fibroblasts, the possibility should

not be excluded that in the absence of certain specific factors, such as hormones, the metabolism of cells tends to revert to a simple standard pattern, adapting itself to the utilisation of whatever energy substrate is presented to it.

SUMMARY

1. 15-day chick embryonic liver explants were grown for up to six days *in vitro*. Metabolic measurements were made immediately after explantation and then at intervals.

2. Immediately after explantation lactate disappeared from the medium while hexose and pyruvate were produced. Within 24 hours this metabolic pattern was reversed and lactate was then synthesised at the expense of available hexose.

3. The initial rate of oxygen utilisation was high, but it decreased rapidly within 24 hours and remained at a relatively low level. It was unaffected by renewing the medium.

4. The specific activity of acid-soluble phosphorus on renewal of the medium after 48 hours *in vitro* rose to a level considerably higher than in the fresh tissue. The specific activity of lipid phosphorus was boosted by the renewal of medium but did not rise to the initial level. The specific activity of the DNA phosphorus fraction decreased during 24 hours after explantation but increased above the original level on renewing the medium.

5. Possible reasons for the complete reversal of the *in vivo* pattern of metabolism on maintenance of liver tissue *in vitro* are discussed.

We should like to express our appreciation to Professor J. N. Davidson for making facilities available to us within the Biochemistry Department, and to Miss Ina Withers for her most efficient technical assistance. The work was supported by grants from the Scottish Hospital Endowments Research Trust.

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THE REACTION OF OSMIUM TETROXIDE WITH TISSUE COMPONENTS

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Osmium tetroxide is almost universally regarded as an excellent fixative as far as preservation of cellular structure in life-like condition is concerned [6, 4, 17]. The range of its use was widened when it was found that fixation in OsO_4 solutions is almost a prerequisite for obtaining sharp electron micrographs [6, 14, 2]. Besides, OsO_4-oxidant mixtures can serve to differentiate degenerating myelin sheaths from normal ones in the Marchi type of staining methods. It has been shown in previous reports that the staining of degenerating myelin is probably due to the liberation of an acid polysaccharide during the degeneration [18, 19].

This study was undertaken in order to elucidate the nature of the effects of OsO_4 on tissue constituents, including the mechanism involved in the Marchi type of staining of degenerating myelin by OsO_4-oxidant mixtures.

MATERIAL AND METHODS

Most of the experiments were carried out on rat tissues. Lightly anaesthetized rats were killed by bleeding, and in most experiments pieces of various tissues were fixed in Carnoy's fluid, embedded in paraffin, and cut at 8 μ. In one experiment pieces of the organs were fixed in 10 per cent formalin for 2 days, followed by immersion in Swank and Davenport's [15] solution (containing 60 cc of 1 per cent $KClO_3$, 20 cc of 1 per cent OsO_4, 1 cc of glacial acetic acid, and 12 cc of 37 per cent formaldehyde) for 10 days. Other pieces of the same organs were put directly into Swank and Davenport's solution for 10 days. After rinsing for 24 hours in running water, the pieces were embedded in carbowax (1 part of carbowax 4000 and 4 parts of carbowax 1540 according to a previously described technique [20]).

The sections were either mounted without any counter-staining, or stained by H. and E., or else they were stained with Schiff's leukofuchsin (to visualize carbonyls).

In order to test the reactivity range of OsO_4 solutions, smears of pure chemicals were made on slides marked and divided into squares with a diamond pencil. Crystalline bovine albumen (Armor, Chicago) dissolved in water served as an emulsifying medium for sticking the compounds to the slides. After drying in air the slides were slightly warmed on a Bunsen flame to coagulate the albumen. An alternative method for attaching the compounds to glass slides consisted in putting the com-

pounds between two drops of dilute collodion and hardening of the collodion in 70 per cent ethanol. The following compounds were tested:

Glycogen (Nutr. Biochem. Corp., Cleveland, Ohio); gastric mucin (Wilson, Chicago); Na alginate (B.D.H., England); chitin (Nutr. Biochem. Corp.); cellulose powder (Balston Comp., England); heparin (Bioquimicas Reunidas, Uruguay); hyaluronic acid (originating from Dr. K. Meyer's laboratory); DL sphingosine (synthesized by Dr. D. Shapiro of the Weizmann Institute, Rehovoth, Israel); triacetyl DL sphingosine (idem); sphingomyelin (kindly supplied by Prof. E. Klenk of Cologne); agar (Difco); dextran, native (kindly supplied by Dr. C. R. Ricketts of Birmingham, England); dextran sulphate (idem); nonanoic acid (Eastman Org. Chem., Rochester); ricinoleic acid (idem); and control—albumen only.

Easily soluble pure compounds were tested for their reactivity versus OsO_4 by putting small amounts of the compounds at the bottom of small clean test tubes and pouring 0.5 ml of the osmium solutions over them. The tubes were tightly stoppered and left standing at room temperature for up to 10 days. The following compounds were tested in this way:

Glucose (Nutr. Biochem. Corp.); galacturonic acid, alpha D. (idem); glucosaminic acid (idem); glucosamine hydrochloride (Bios Lab., New York); and control—containing only the OsO_4 solution.

As in some test tube experiments there was blackening of the test tube walls above the solutions, while the solutions themselves remained clear, the same compounds were tested in two ways for their reactivity towards the OsO_4 solutions: (*a*) the compound tested was delivered on the side of a tilted test tube which contained the osmium solution. On straightening the tubes, the area where some grains stuck to the wall was marked on the outside of the tube with a grease pencil; (*b*) the compound to be tested was delivered in concentrated solution or suspension to the bottom of the tube, so as to avoid any dry particles getting attached to the wall of the test tube.

The tested OsO_4 solutions included, besides OsO_4 in water and the above-mentioned Swank and Davenport's solution, also 1 per cent OsO_4 in anesthetic ether, and 1 per cent OsO_4 in carbon tetrachloride.

EXPERIMENTAL RESULTS

Rat tissue sections.—Rat tissues which were fixed in formalin for 2 days and then exposed to the action of Swank and Davenport's solution (an OsO_4-oxidant mixture) for 10 days did not differ appreciably from those obtained with tissues immersed at once in Swank and Davenport's solution or in 1 per cent OsO_4 solution. In both cases only structures which are presumed to contain lipids (such as fat cells and droplets, Golgi apparatus in the intestine, mitochondria in the renal tubules) were stained black in the sections. Myelin sheaths, and to a lesser extent the liver and kidney cells, stained pale yellow. Staining of such sections with hematoxylin and eosin revealed that acidophilia of all tissue constituents was abolished. The staining of basophilic

uctures with hematoxylin did not seem to be affected except for the in-tinal tract mucus which failed to stain. The myelin sheaths failed to stain her with hematoxylin or with eosin.

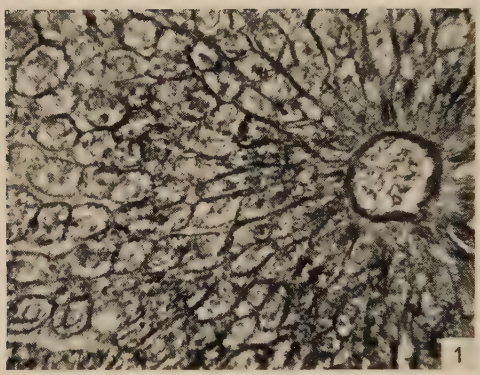
In the following series of experiments sections of rat tissues which were ed in Carnoy's fluid were exposed to the action of OsO_4 in non-aqueous utions. The results obtained with OsO_4 dissolved in carbon tetrachloride re similar to those obtained with OsO_4 in ether, but as the etheric solution on deteriorated (with the appearance of copious black precipitate), the Cl_4 solution was found preferable. Immersion of sections for 24 hours in her of these solutions, followed by 2–3 rinses in the solvent, clearing in lene and mounting in balsam yielded the following results:

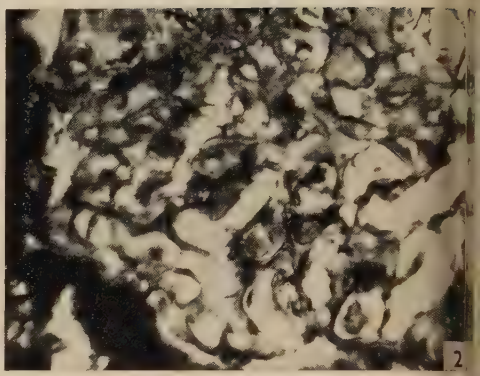
In all the tissues there was a distinct brown-black staining of the cell mbranes, and often also of the nuclear membranes (Fig. 1). Mitochondria the heart, liver and kidney cells were also often stained. In striated muscles d in the myocardium longitudinal and transverse striations were stained most places. In the renal glomeruli double basement membranes could observed in many places, similar to those visualized in electron micro-pical sections (Fig. 2). In the brain, the nerve-cell cytoplasm and the ons were stained brown. Gastro-intestinal and bronchial mucus showed egular staining characteristics. The cytoplasm of the gastric parietal cells ined in many places brown-black (Fig. 3). In the spleen, besides the stain-g of the reticulum fibres, the cytoplasm of the megakaryocytes was also ined deep brown.

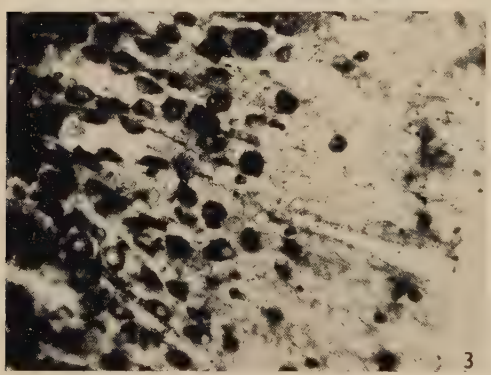
In order to test the possibility that carbonyl groups might be formed as a ult of OsO_4 oxidation, slides were exposed to the action of OsO_4 in ether or Cl_4 as above, rinsed twice in the solvent, dried, and transferred for 1 hour o a 2 per cent solution of potassium chlorate in water for decolorizing. ter thorough rinsing in water, the slides were stained by Schiff's leuko-chsin for 15 minutes, followed by the usual sulfite treatment (as in the A.S. procedure). Control slides, which were put directly into the $KClO_3$ lution followed by water rinse and leukofuchsin, did not stain with leuko-chsin at all.

The appearance of the sections stained by leukofuchsin (after the osmium ecipitate was removed) was somewhat similar to the appearance of sections ined by the P.A.S. method, but basically it was an inferior quality repetition the original osmium staining: The basement membranes in the kidney re stained pale red, while in the liver cells the glycogen stained very pale d, and the cell membranes stained somewhat more intensely.

Deparaffinized sections of rat tissues fixed in Carnoy's fluid were exposed







The three figures are of rat organs fixed in Carnoy's fluid, embedded in paraffin and stained with OsO_4 1 per cent in CCl_4.
Fig. 1. Liver. Cell membranes, nuclear membranes and often mitochondria are demonstrated. × 345.
Fig. 2. Kidney. Double basement membranes are seen in the glomerular tuft. × 715.
Fig. 3. Stomach. Dark staining of the parietal cells. Staining of membranes of other cells is also visible. × 270.

also to the effect of OsO_4 vapours or vapours of Swank and Davenport's solution, by leaving the slides for 24 hours in a tightly stoppered staining dish containing a few cc of either of the solutions. The staining obtained was similar to that obtained by treatment with OsO_4 in CCl_4, although less regular and less intense. The only clear-cut difference between the two treatments by the vapours was observed in muscle tissue: in sections stained with vapours of the Swank–Davenport solution, muscle tissue stained dark brown to black and could be well differentiated from connective tissue which stained pale brown. In the sections treated by OsO_4 vapours both muscle and connective tissue stained pale brown.

Pure compounds.—Table I summarizes the results obtained with pure compounds treated by the various osmium solutions. The findings with Swank and Davenport's solution corroborate the author's previous findings [18], and in addition the test tube experiments show that the OsO_4 solution reacts with aminosugars producing black droplets on the wall of the test tube above the liquid, while the liquid phase remains clear.

TABLE I

Effect of various OsO_4 solutions on pure compounds.

pound	I Swank–Davenport's solution for 7 days	II 1 % OsO_4 in CCl_4 or 1 % OsO_4 in anesthetic ether for 24 hrs	III As II, followed by 1 hour in 2 % $KClO_3$ rinse and leukofuchsin
	On slides	On slides	
cogen	0	0	±
tric mucin	+ +	+	+
alginate	0	0	+ +
in	0	+ +	+ +
ulose powder	0	0	0
arin	+ +	+ +	?
luronic acid	+ +	+ +	+
phingosine	+ +	+ +	+ +
cetyl DL sphingosine	0	0*	0*
ingomyelin	+ +	+ +	+ +
r	0	+ +	+ +
tran, native	0	0	+
tran, sulphate	0	0	+
anoic acid	+ +	0*	0*
noleic acid	+ +	0*	0*
trol	0	0	0
	In test tubes	On slides	On slides
ose	s 0; w 0	0	±
cturonic acid	s 0; w 0	0	0
osaminic acid	s 0; w +	±	±
osamine	s 0; w + +	+	+
trol	s 0; w 0	0	0

ntensity of staining was graded as follows: 0 = no staining; ± = pale yellow or (in the leukosin-stained sections) pale pink; + = brown, or red respectively; + + = black, or purple ectively.

n the test tube experiments: s = color of solution; w = color of droplets on wall above the tion.

he * indicates that the result yields no real information as the compound was dissolved pletely during the staining.

On the whole, solutions of OsO_4 in CCl_4 or in ether behaved similarly to solutions of OsO_4 in water in the slide experiments. An important difference was noted only in regard to poly-aminosugars (chitin and agar) which stained after treatment by OsO_4 in the non-polar solvents and not after treatment with Swank and Davenport's solution.

TABLE II

Effect of OsO_4 solutions on pure compounds in solution and in dry form.

Compound	1 % OsO_4 in dist. water 24 hrs		Swank–Davenport's solution 24 hrs	
	Solution*	Particles adhering to wall	Solution*	Particles adhering to wall
Glucosaminic acid	black	black	yellow	black
Glucosamine	0	0	0	black
Na alginate	0	0	0	0
Chitin	0	0	0	0
Heparin	pale yellow	0	0	black
Agar	0	0	0	brown
Glycogen	0	0	0	0
Control	0	—	0	—

* The results obtained when the compounds tested were delivered in solution or suspension to the bottom of the test tubes were similar to those obtained in the dissolved part of the compounds when the compounds were delivered in powder form.

The results of staining of the compounds oxidised by OsO_4 in non-polar solvents with leukofuchsin indicate that all the polysaccharides tested (with the exception of cellulose) were oxidized, although to different degrees, by the osmium solutions.

Table II shows that glucosamine, heparin and agar when in particulate form are blackened by the Swank–Davenport's mixture and not by OsO_4 alone. The table also summarizes the results of the experiments intended to clarify the meaning of the black droplets on the walls of test tubes which contained glucosamine and glucosaminic acid. It is clear from the results that the black droplets observed on the walls are due to small particles of the compounds which became attached to the walls of the tubes.

DISCUSSION

The experiments in which tissue sections were exposed to the action of aqueous OsO_4 solutions confirm the known fact that OsO_4 solutions abolish acidophilia of tissue constituents. As acidophilia of tissue constituents is known to depend on amino groups [12, 21], it is clear that OsO_4 blocks or destroys the basic amino groups as has been shown by a number of observers [11, 5, 2].

Basophilia was reduced or abolished only in the case of mucus, while t due to RNA and DNA was not apparently affected. This effect indicates t in the case of mucoid substances OsO_4 reacts not only with the amino ups, but also with the acidic groups present and destroys or blocks them. The appearance of tissue sections treated by OsO_4 in non-polar solvents icated that a brown-black precipitate of osmium was formed on cell-walls d often on the walls of nuclei and mitochondria. These sections looked ilar to electron micrographs of tissues fixed in OsO_4 and revealed the sence of double basement membranes in the glomerular tufts of the kidney. t has been pointed out [3] that the contrast obtained in electron microscopy OsO_4-treated sections is due mainly to the deposition of osmium. On the er hand, as is well known (and has been confirmed in this study), sections ated by aqueous solutions of OsO_4 do not show blackening of the cell mbranes and other structures which are visible under the electron micro-pe. It is probable that osmium is present in tissues fixed in OsO_4 in a ourless form, while brown black precipitates form when the reaction urs in non-polar solvents. The similarity observed between sections of ue treated by OsO_4 in non-polar solvents and electron micrographs of ues fixed in OsO_4, depends probably on the fact that while the electron am is sensitive to the presence of osmium in any form, light microscopy ws only the dark osmium precipitates. These are formed by OsO_4 in n-polar solvents, and not by aqueous solutions of OsO_4.

The results obtained with the pure compounds indicate that OsO_4 reacts h a great variety of chemical groups. Three types of reactions have been served to occur between OsO_4 and the compounds tested.

(1) Reactions resulting in the formation of a black precipitate when the npounds are treated by watery solutions of OsO_4. This type of reaction urs between OsO_4 and unsaturated lipids as well as with compounds ntaining various strongly reducing groups (SH, reducing phenols, cf. [2, 18]).

(2) Reactions in which a black precipitate is formed when the compounds treated by OsO_4 in a non-polar medium, while no black precipitate is med in aqueous OsO_4 solutions. This reaction occurs between OsO_4 and ne amino compounds. The black staining was observed when the com-unds were treated by solutions of OsO_4 in non-polar solvents, and also en granules of the compounds sticking to the walls of test tubes were posed to the action of OsO_4 vapours. Furthermore, the same reaction urred in an aqueous medium in the part of the same compounds (attached a slide) which was not completely hydrated and therefore not dissolved. is fact explains the previously made observation [18] that the staining of

heparin by OsO_4 solutions in water depended on its presence in an undissolved form.

It may be suggested that the type of reaction involved in these cases is as follows. The addition product formed between OsO_4 and the amino compounds in non-aqueous media contains osmium in a reduced form. In aqueous media the formation of the addition products either does not involve any reduction of the OsO_4 or else it might involve primary reduction followed by reoxidation. It may be noted that the formation of dark complexes between OsO_4 and some organic compounds has been postulated by some workers [7, 8, 1]. This type of reaction would explain why no blackening (except for unsaturated fats) is observed in tissues fixed in watery OsO_4 solutions or in *wet* tissues fixed by OsO_4 vapours, while electron microscopy and chemical determination [3] reveal evidence of osmium deposition. A similar reaction was known to occur between aqueous solutions of OsO_4 and formaldehyde [10]; a diffuse blackening of the wall of the test tube occurs long before the solution becomes dark. The usual explanation given for this phenomenon [9] is that formaldehyde gas is more reactive than the solution. In view of the fact that OsO_4 reacts rapidly with alcoholic solutions of formaldehyde and forms a dark precipitate [10, 13], it is probable that OsO_4 reacts with some aldehydes in a way which is similar to that described above for some amino compounds.

(3) Reactions in which the compounds are oxidized by OsO_4 without the formation of an osmium-containing precipitate. Polysaccharides, and presumably therefore all compounds containing 1–2 hydroxyls, are oxidized by OsO_4 in non-polar solutions to carbonyl-containing compounds in a way similar to that obtained by periodic oxidation. This reaction, described by Criegee [7], was shown to occur in tissue sections which have been stained by leukofuchsin. The oxidation might not stop at the aldehydic stage when aqueous solutions are used. As glycogen deposits are not known to be visualized in OsO_4 fixed material examined with the electron microscope, it is possible that in this case the OsO_4 serves as an oxygen carrier, being reduced to an easily oxidizable form, which is subsequently reoxidized by the atmospheric oxygen present in the solution. The free, volatile OsO_4 is not kept by the tissue.

The foregoing discussion indicates that the appearance of black colour cannot be regarded as a criterion indicating that a reaction occurs between OsO_4 and various compounds, as in many instances the products of the reaction are colourless. A further study of the reactions occurring between OsO_4 and various organic materials is obviously needed.

Table II shows that particles adhering to the walls of test tubes are differently affected by the presence of OsO_4 and OsO_4-oxidant solution at the bottom of the tube. The introduction of a filter paper wetted with KI and starch solutions, into a closed test tube containing Swank–Davenport's solution showed that the atmosphere above the solution contains ClO_2 in addition to OsO_4 as the filter paper became blue. It is obvious, therefore, that the OsO_4-oxidant mixtures can act in a way different from that of OsO_4 alone, also in non-aqueous medium. This finding might be of major importance for the explanation of the Marchi type of stains for degenerating myelin, in which OsO_4-oxidant mixtures are used. While OsO_4 alone stains normal and degenerating myelin equally, these mixtures stain degenerating myelin to the almost complete exclusion of normal myelin. It has been shown [18] that for most compounds known to react with OsO_4, the blackening is either unmodified or less intense with OsO_4-oxidant mixtures as compared to OsO_4 alone. The only compound which behaved like a "Marchi type" compound, i.e. which was blackened more intensely by OsO_4-oxidant mixtures than by OsO_4 alone in those experiments, was heparin. The present experiments indicate that the moiety responsible for this type of reaction is glucosamine (presumably any hexosamine), and that the liberation of hexosamine-containing compounds during demyelination is therefore probable.

SUMMARY

A study was made of the effects of OsO_4 in aqueous and non-polar solutions on tissue sections and on some pure compounds.

Aqueous solutions of OsO_4 inhibit the acidophilia of tissue constituents and the basophilia of gastro-intestinal mucus. Treatment of tissue sections by OsO_4 in non-polar solvents results in a histological picture which is similar to that obtained in electron micrographs of tissues fixed in solutions of OsO_4.

The evidence presented indicates that OsO_4 reacts with tissue constituents in at least in three different manners: (*a*) with some compounds black precipitates are formed in aqueous solutions; (*b*) with other compounds black precipitates are formed only in the absence of water or with incomplete hydration; (*c*) with still other compounds which react with OsO_4, no precipitation of osmium occurs.

Glucosamine (and glucosamine-containing polysaccharides) were found to be compounds which in the non-hydrated state form black precipitates with OsO_4-oxidant mixtures and not with OsO_4 alone. It is considered probable

that a hexosamine-containing compound is made available for reacting during demyelination and is responsible for the Marchi staining.

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METHOD FOR STAINING TISSUE AND CELL MEMBRANES

ODUCING HISTOLOGICAL PICTURES SIMILAR TO THOSE OBTAINED BY CURRENT ELECTRON MICROSCOPY METHODS

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E simple staining method described in this paper results in a staining of l and other membranes giving a picture similar to electron micrographs of OsO_4-fixed tissues. The present paper deals with a description of the staining thod and its results, as well as with an attempt to explain its chemical sis and the reason for the similarity with electron micrographs of OsO_4-ed tissues.

THE STAINING METHOD

Hydrated sections of tissue which were fixed in Carnoy's fluid and embedded in affin are immersed for 10 minutes in a 1 per cent solution of mercuric acetate in tilled water. After three rinses (about 1–2 minutes each) in distilled water, the tions are left to dry. They are then exposed for 2 minutes to H_2S gas in a closed lin jar which contains a few cc. of ammonium sulfide at the bottom. The sections rinsed in water, dehydrated and mounted in Canada Balsam. The sections may counterstained with Kernechtrot, eosin, van Gieson, or the P.A.S. method.

RESULTS

Staining of tissue sections.—In rat tissues the best results were obtained with tions cut at 2 μ. Thicker sections were often hard to interpret because of intense staining.

n the *brain* the membrane of nerve cells was stained black and could be lowed in the neurite, where it was found to envelop the myelin sheath g. 1). The membrane continued into the white substance, always at the er surface of the myelin sheaths (Fig. 2), delimiting the neuronal process m a weakly stained network of ground substance.

In the *myocardium,* the staining demonstrated both longitudinal and nsverse striations. In addition, mitochondria and nuclear membranes re also stained.

n the *bronchial epithelium* the cell membranes were well demonstrated.

A black membrane was found around the mucus droplets, and an additional membrane was found on the luminar surface of the cells (Fig. 3). This membrane, situated at 0.5–1 μ beyond the cell membrane proper, formed a continuous layer over the lumen, with projections covering the cilia, and was interrupted only at the mouths of the mucus cells.

In the *lungs* the interalveolar septa were lined by what appeared as a single membrane. In some places (Fig. 4) the membrane could be resolved into two separate lines.

In the *intestine*, the epithelium showed a structure similar to that of the bronchus. The lumen and the lumen of the glands were delimited by two membranes, the cell membrane proper, and an outer membrane (Fig. 5). The outer membrane was interrupted at the openings of goblet cells, while the cell membrane also delimited the mucus droplets from the cell bodies.

In the *liver*, the staining of the cell membranes could be seen best. The limit between two contiguous liver cells could be seen to be formed from two distinct lines, the respective membranes of the two cells, with a narrow clear interstice between them (Fig. 6). A similar double membrane could be seen lining the sinusoids, with a capillary membrane lying on top of the liver-cell membranes. In addition, Fig. 6 shows that the nucleus is delimited from the cytoplasm by a membrane which in some cells appears to be double. It seems that both the cytoplasm and the karyoplasm are delimited by membranes at this interface. In many cells membranes of nucleoli and mitochondria could also be seen.

In the *spleen* (Fig. 7), the sinuses were found to be lined by a membrane. In some places, where the intersinusoidal reticulum cells were situated directly beneath the sinus membrane, the membrane appeared double with the cell membrane and sinus membrane situated near each other.

In the *kidney*, the double membrane of the glomerular tuft was stained clearly (Fig. 8). A weakly staining mesangium was delimited by the capillary membrane, while the outer surface of the loops showed a capillary membrane and 0.5–1 μ beyond it, the epithelial membrane. Sections counterstained with the P.A.S. method showed that the P.A.S. staining material was situated between the two membranes.

In the kidney tubules the appearances were essentially similar to those found in the liver. Cell membranes, capillary membranes and nuclear membranes were stained (Fig. 9). In many places mitochondrial outlines were demonstrated.

In a medium-sized *artery* (Fig. 10) the elastica could be seen as an empty band surrounded on both sides by thin black membranes.

In the other rat organs examined, the findings were basically similar to those described above. Collagen was not stained anywhere.

Staining of pure materials.—In order to test the reactivity range of the method, pure compounds were put between two layers of dilute celloidin on diamond-marked slides. Full particulars on the compunds were given in the preceding article [4].

In order to test the reactivity of water-soluble compounds, the staining procedure was changed by substituting a saturated solution of mercuric acetate in chloroform for the aqueous solution of this salt. The slides containing the tested compounds were rinsed in three changes of chloroform after the mercuric treatment and exposed to H_2S gas after drying. The results of both series of experiments with the pure materials are given in Table I.

Effect of variations in the technique.—Attempts were made to determine the effects of various fixatives. In human and rat tissues fixed in formalin or in Zenker's fluid (the sections of the Zenker-fixed material having been treated with an alcoholic Lugol solution, as usual, for dissolving the mercuric chloride precipitates), the staining yielded similar but less satisfactory results than after Carnoy fixation.

Rat tissues fixed in Zenker or in a saturated solution of mercuric chloride were exposed directly after deparaffinization to H_2S vapours. No staining of membranes was obtained. Carnoy-fixed tissues exposed to the action of mercuric chloride could not be stained. On the other hand, mercuric nitrate was quite satisfactory as a 1 per cent solution, as subsequent treatment by H_2S vapours resulted in good staining of membranes.

Rat tissues were fixed for 24 hours in a 10 per cent solution of mercuric acetate, rinsed and embedded in paraffin. The deparaffinized sections were exposed to the action of H_2S. Good staining of membranes was obtained, but fixation was not as satisfactory as that obtained with Carnoy's fluid.

The procedure in which the materials do not come in contact with water could be applied also to tissue sections. Paraffin blocks were cut and the sections floated on 70 per cent ethanol and dried. After deparaffinization, the sections were treated in the same way as the slides containing the pure compounds which are water-soluble. The staining was similar although less intense than the staining by the usual procedure.

Two more technical details may be noted: (1) The reason for exposing the sections to H_2S gas rather than to a solution of ammonium sulfide is the solubility of the HgS in ammonium sulfide. Sections treated by a solution of $(NH_4)_2S$ were stained irregularly or not at all. Dryness of the sections exposed to the hydrogen sulfide is therefore essential. (2) The deparaf-

TABLE I

Staining of pure compounds smeared on slides by the mercuric sulfide method.

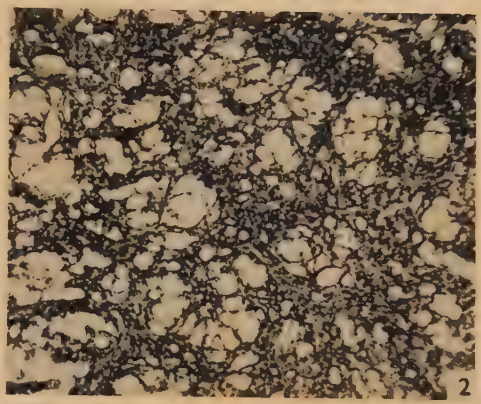
Compound	Intensity of staining*
(a) Compounds treated by an aqueous solution of mercuric acetate	
Glycogen	0
Gastric mucin	+
Na alginate	+
Chitin	+ +
Cellulose powder	0
Heparin	+
Hyaluronic acid	+ +
DL Sphingosine	+
Triacetyl DL Sphingosine	0
Sphingomyelin	+ +
Agar	+ +
Dextran, native	0
Dextran sulphate	+ +
Nonanoic acid	±
Ricinoleic acid	+ +
Control	0
(b) Compounds tested by a chloroform solution of mercuric acetate	
Glucose	0
Galacturonic acid	±
Glucosaminic acid	+ +
Glucosamine	+ +
Control	0

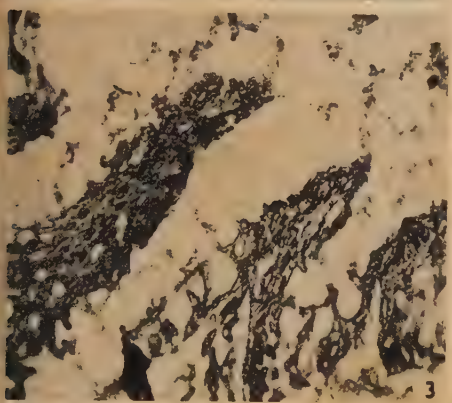
* Intensity of staining was graded as follows: 0 = no staining; ± = very weak or questionable staining (yellowish); + = fair staining (brown); + + = intense staining (black).

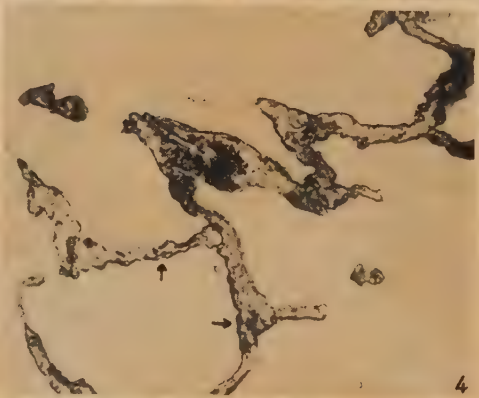
finization of sections had to be complete. A thin layer of undissolved paraffin could efficiently disturb the penetration of the H_2S in parts of the sections.

DISCUSSION

From a morphological viewpoint, the method shows a number of details which could until now only be seen with the aid of the electron microscope. The presence of distinct cell membranes is demonstrated for animal cells, and in the liver, for example, the border between two cells is shown to consist of three separate elements: two cell membranes and between them



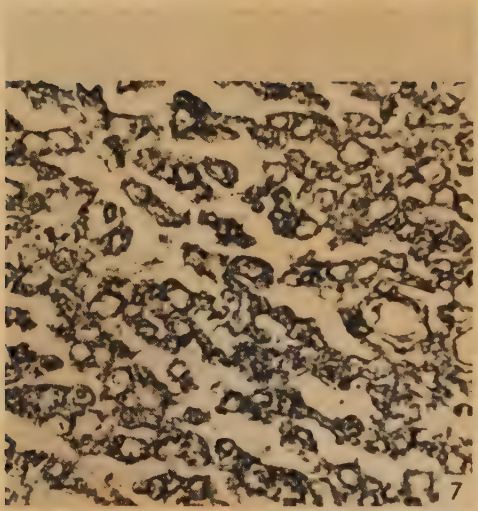




All figures are of sections of rat tissue, 2 μ thick, stained by the mercuric sulfide method as cribed in the text.

. 1. Cerebral grey matter. Cell membranes enveloping perikaryon and continuing on the outer face of the myelin sheaths. × 400.

. 2. Cerebral white matter. Membranes are situated on the outer surface of the myelin sheaths, l between them is a mesh of non-neuronal substance (probably ground substance containing il processes). × 400.



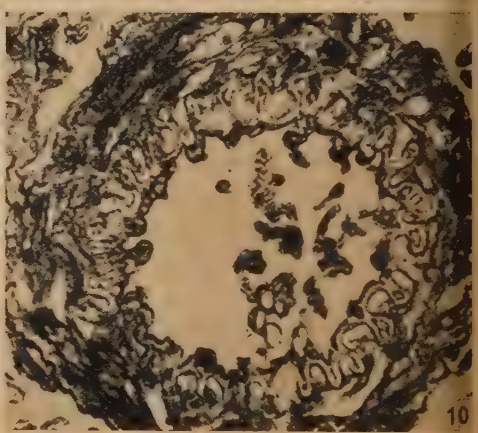


Fig. 3. Bronchial mucosa. Cell membranes and outer membrane. The outer membrane envelopes the cilia and is interrupted at the openings of goblet cells. × 400.
Fig. 4. Interalveolar septa of lung. Septa lined by a membrane which appears double in some places (arrows). × 400.
Fig. 5. Intestinal mucosa. Cell membranes and outer luminar membrane interrupted at the openings of goblet cells × 400.
Fig. 6. Liver. Cell borders consisting of two distinct membranes belonging to contiguous cells with a thin unstained interspace between them. A double nuclear membrane can be seen in some cells. Nucleoli and mitochondria. Enlarged appr. × 2 diameters from magnification × 375.
Fig. 7. Spleen. Cell membranes and sinusoidal membranes. Enlarged appr. × 2 diameters from magnification × 375.
Fig. 8. Renal glomerulus with double basement membranes. × 1070.
Fig. 9. Renal tubules. Cell membranes, nuclear membranes and mitochondria. Double membrane along line of contact between tubules and capillaries. × 1150.
Fig. 10. An artery in the kidney. The lamina elastica interna is enveloped on both sides by membranes. Enlarged appr. × 2 diameters from magnification × 400.

hin layer of intercellular material. The presence of a limiting membrane, lependent of cell membranes, over the mucosal surfaces might be of erest in the study of the mode of penetration of absorbed food and bacteria o the tissues from the lumen. In the brain, the findings seem to indicate it the myelin sheath is an integral part of the neurone, lying within its rders, rather than a product of secretion produced by other cells.

In the kidney, the double basement membrane of the glomerular tuft, th a third intermembranous layer between the two, was surmised but uld not be shown with the optical microscope [2].

In the lung and at the border between nucleus and cytoplasm a single mbrane was observed in most places, but a double membrane could be tinctly seen in a few areas. It is believed that the membrane is double erywhere, but the distance between the membranes is so small that the plicity can be seen only in the places where the two membranes run in nes exactly perpendicular to the section.

The question whether or not the staining of the various membranes should considered as an artefact can be answered in accordance with a previously pressed line of reasoning [5]. The staining indicates that chemical groups pable of binding mercury are distributed along the membrane surfaces ich are stained. While it is possible that these groups may belong to thread- e molecules extending far beyond the membranes, the membranes defi- ely exist in both a chemical and morphological sense as distinct surfaces which these groups are abundant.

The results of the experiments done on pure compounds and summarized Table I indicate that amino-sugars and unsaturated compounds with ctive ethylenic links are stained intensely by this method. This is not prising in view of the known affinity of these mercuric compounds for ylenic links [1] and of the known reactions between heavy metals and ine groups [5]. Without reference to the experimental data reported here, may also be assumed on the basis of common knowledge that sulphydryl ups will also be stained by this method. The three above-mentioned mical groups also react with OsO_4. This fact might explain why the ining obtained with this method is similar to the staining obtained by O_4 in non-polar solvents [4] and in the electron micrographs of OsO_4- ed tissues. It may be noted that from a morphological point of view the ining by this method results in much clearer detail than that obtained by O_4 in carbon tetrachloride.

Besides the above-mentioned chemical groups, Table I indicates that also dic groups and especially strongly acid ones are stained by this method.

SUMMARY

A simple method of staining membranes which results in the deposition of mercuric sulfide is described. Tissue sections stained by this method show, among other things, cell and nuclear membranes, an outer membrane lining mucous surfaces, and a double membrane enveloping the glomerular tuft of the kidney.

Study of the types of compounds which are stained by the method affords an explanation of the similar appearances obtained by this method, by OsO_4 in non-polar solvents, and by OsO_4 fixation in electron micrographs.

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HE COMPARATIVE NUCLEIC ACID CONTENT OF LIVER JCLEI, OVA NUCLEI AND SPERMATOZOA OF THE FROG[1]

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HE Boivin–Vendrely theory of the constancy of the DNA content in loid somatic non-dividing nuclei, this quantity being double that found haploid cells, was based upon the experimental results of Boivin *et al.* [1] d Vendrely and Vendrely [13] and received confirmation by Mirsky and s [4, 5]. Since then numerous investigators have published reports on the NA content of the diploid somatic cells of various tissues of animals of veral species. The spermatozoa of the fish, the fowl and of several mam- alia provided the data substantiating the concept that the DNA content of ploid cells was one-half that of diploid somatic cells.

Investigations employing ova have yielded data which, to date, have ntributed little information in conformance with the Boivin–Vendrely ory. Since analyses of mammalian ova are not practicable, the chemical alyses for DNA have been made on the ova of several invertebrates by hmidt *et al.* [5], Vendrely and Vendrely [19], and others. The eggs of one rtebrate, the frog, was investigated by Hoff-Jørgensen and Zeuthen [3]. all of these analyses for the DNA content of ova the whole cell was em- oyed and the investigators reported that the whole egg of these species ntains from several hundred to 5000 times the DNA found in the sperma- oa of the same species. No reports on the DNA content of the nuclei of a obtained by chemical analyses are available.

It is the purpose of this paper to report the results of chemical analyses of a nuclei, spermatozoa, and liver nuclei of the frog in which DNA, RNA, d arginine have been determined. The arginine determinations were made a preliminary approach in an investigation of the nature of the proteins spermatozoa and ova of the same species.

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MATERIALS AND METHODS

Mature frogs (*Rana pipiens*)[1] were used as a source of the ova, spermatozoa, and liver cells in this investigation.

Isolation of ova nuclei.—Four different methods were utilized for the isolation of ova nuclei during the preliminary experimental stages, including (*a*) a microdissection method, (*b*) one in which 1 per cent citric acid solution and the Waring blendor were employed, (*c*) a method utilizing inert sucrose solution of 0.88 *M* concentration as the homogenizing medium, and (*d*) the method finally selected in which 70 per cent sucrose solution constitutes the homogenizing medium.

The 70 per cent aqueous sucrose method yielded better preparations from which the cytoplasm is easily separated by washing and during which no agglutination, either of nuclear or cytoplasmic material, occurred. Prior to the homogenization of the frog ova in the sucrose medium, the cells were immersed in cold 50 per cent trichloroacetic acid for a period of three to five minutes. The trichloroacetic acid treatment hardened the cells which were then easily broken by a three minute homogenization period. The nuclei were isolated by washing and centrifuging with 70 per cent sucrose solution as the suspension medium. The isolated nuclei were resuspended in cold 10 per cent trichloroacetic acid solution and the number of nuclei per ml of suspension determined with a haemocytometer. The nuclei were ruptured prior to chemical analysis with a Potter-Elvehjem glass homogenizer.

Isolation of frog spermatozoa.—The testes, at least 70 mm in length, were removed immediately after slaughter of the frogs, and were washed and crushed in saline solution with a Potter–Elvehjem homogenizer. Connective tissue and other cells in the spermatozoan suspension were removed by straining through double bolting cloth, then through a 200 mesh wire screen. Differential centrifugation in distilled water resulted in the isolation of the spermatozoa. Counts of the spermatozoa in the suspension used for the chemical analyses were made in a haemocytometer chamber.

Isolation of liver cell nuclei.—Fresh livers from the frogs were broken in a Waring blendor with 70 per cent sucrose solution, containing crushed ice, as the homogenizing medium. After a homogenization period of ten minutes, rather pure nuclei were isolated by differential centrifugation.

Chemical analyses.—One ml of the homogenate of the nuclear preparations were utilized for the extraction procedures for the separation of acid-soluble phosphorus, phospholipid, phosphoprotein, and nucleic acid fractions by methods based on those described by Schmidt and Thannhauser [9] and Schneider [8].

The total acid-soluble phosphorus was determined as inorganic phosphorus by the method of Fiske and Subbarow [2]. This method and that of Youngburg and Youngburg [15] were the basis of the procedure utilized in the determination of lipid phosphorus.

The method used for the colorimetric determination of the DNA content of the nuclei was essentially that described by Schneider [8] with a few minor modifications. The Schneider method utilizes the Dische diphenylamine reaction in the colorimetric analysis of DNA. The method described by Schneider [8], in which orcinol is the

[1] The authors are indebted to Dr. John R. Shaver, Experimental Embryology Research Laboratories, Zoology Department, University of Missouri, for supplying the frogs.

or-forming agent, was employed for the PNA analyses. Again a slight modification he procedure described by Schneider was necessary. The quantity of the PNA tion was increased in order to increase the intensity of the color formed with the gent prior to measurement in the colorimeter. As a check on the results obtained, mited number of nuclei samples were fractionated by the method of Ogur and sen [6].

he nitrogen content of the nuclei was determined by a micro-Kjeldahl, steam illation procedure. The samples were digested with sulfuric acid and metallic nium as the catalyst. After the addition of NaOH solution to the digest, the am- nia is steam-distilled into a receiving flask containing 2 per cent boric acid and indicator. Titration of the contents of the receiving flask is made with 0.02 *N* sul- c acid.

he procedure used for the arginine analyses was based on the method reported Thomas *et al.* [11].

RESULTS AND DISCUSSION

n order that comparisons may be more readily visualized, the data for the analyses of the nuclei of the three different cell types of the frog have n presented in a single table (Table I). It is well to remember that the iable of most importance, in respect to the theory of the constancy of A, is the number of sets of chromosomes in each of the nuclei studied. ce the eggs of the frog were collected from the abdominal cavity prior

Table I

mary of results of chemical analyses of the nuclei of frog ova, spermatozoa and liver cells. values are averages and are expressed in picograms* per nucleus ± one standard deviation.

tion analyzed	Ova nuclei	Spermatozoa	Liver nuclei
id-soluble phosphorus	Negative	Negative	Negative
Lipid-P	1.28 ± 0.2 (7)**	0.9 ± 0.1 (7)	1.51 ± 0.2 (13)
DNA	13.27 ± 1.0 (11)	6.48 ± 1.0 (16)	15.76 ± 1.0 (13)
DNA-P	1.31 ± 0.01 (11)	0.64 ± 0.2 (16)	1.56 ± 0.1 (13)
PNA	1.13 ± 0.2 (3)	0.93 ± 0.7 (10)	0.68 ± 0.2 (13)
PNA-P	0.11 ± 0.03 (3)	0.09 ± 0.01 (10)	0.065 ± 0.01 (13)
DNA-N	2.22 ± 0.4 (11)	1.09 ± 0.3 (16)	2.64 ± 0.3 (13)
PNA-N	0.19 ± 0.1 (3)	0.146 ± 0.03 (10)	0.11 ± 0.01 (13)
Protein-N	6.25 ± 0.3 (2)	4.3 ± 1.0 (14)	20.27 ± 6.0 (11)
NA-N	2.58 ± 0.2 (3)	1.09 ± 0.3 (10)	2.76 ± 0.3 (13)
Protein/DNA	2.65 ± 0.1 (2)	4.5 ± 1.0 (13)	8.20 ± 2.0 (11)
Arginine	4.7 ± 0.7 (7)	4.4 ± 2.0 (16)	4.6 ± 1.0 (16)
Arginine/DNA	0.354	0.680	0.292

* One picogram is equivalent to 10^{-12} grams.
** Figures in brackets indicate number of samples analyzed.

to the reductional division resulting in the haploid number, one would expect the DNA content to be that of cells containing the diploid number of chromosomes. The quantity of DNA present in these frog ova nuclei is double that found in the frog spermatozoa as shown by the DNA, DNA-N (nitrogen), or DNA-P (phosphorus) content.

Thus, the Boivin–Vendrely theory of the constancy of DNA holds for ova, if ova nuclei rather than the entire cell is analyzed. Actually, the DNA content of the ova nuclei is, within experimental error, nearer the theoretical value of twice that of the DNA content of spermatozoa than is the DNA content of the somatic cell nuclei, the liver nuclei. Sibatani and Fukuda [7] and Wison and Leduc [14] reported the presence of multinucleate cells in the livers of the rat and the mouse. This may be the explanation for the higher DNA content of the liver nuclei. The only other value for the DNA content of frog liver nuclei is that of 15.7, reported by Mirsky and Ris [4], a value identical with that found in this investigation.

There are no data available in the literature on the quantity of DNA of frog spermatozoa; hence, this is the first investigation in which the spermatozoa, ova nuclei, and the nuclei of a somatic cell have been compared on the basis of their nucleic acid content.

The PNA was low in the nuclei of the cells studied, especially in the ova nuclei, and it was more difficult to determine than the DNA, resulting in more variable data.

The arginine determinations were made as an initial endeavor to test a theory that the ova and spermatozoa of a species may differ in the types of nuclear proteins present. The results of the arginine determinations on the frog spermatozoa were quite variable with a coefficient of variation of 45.4 per cent. Therefore, no definite conclusions based on these data are feasible. However, the data obtained would suggest that the proteins of the spermatozoa are of an arginine-rich type. Based on the calculation of the arginine–DNA ratio per set of chromosomes present, it would appear that there is about four times as much arginine per unit of DNA in the spermatozoa as is present in the ova and liver nuclei. Perhaps an arginine-rich protein not associated with DNA may be present.

SUMMARY

Chemical analyses were made on the spermatozoa, ova nuclei, and liver cell nuclei of the frog for DNA, DNA-phosphorus, DNA-nitrogen, PNA, PNA-nitrogen, arginine, and protein nitrogen.

A method in which 70 per cent sucrose solution was used as a homogenization medium for the mass isolation of frog ova nuclei and liver nuclei is developed.

All of the nuclear preparations were found to be free of acid-soluble phosphorus suggesting the absence of protoplasmic contamination.

Results of the DNA analyses provide additional data in confirmation of the Boivin–Vendrely theory of the constancy of DNA in cells of the same species. Further, it is shown that, despite the high DNA concentration found in the cytoplasm of the ova of the frog and several invertebrates, the theory holds for isolated ova nuclei of the frog. The DNA content of ova nuclei of the frog, collected prior to the reductional division to the haploid state, was found to be twice that of spermatozoa.

The DNA content of the nuclei of a somatic cell, liver cells, was slightly greater than twice the spermatozoan content. These results have been discussed.

None of the other nuclear constituents showed a relationship similar to that of the DNA content of the nuclei of the cells investigated.

The spermatozoa of the frog contained 6.48 picograms of DNA per cell. No comparative data on frog spermatozoa are available.

Although the arginine content of frog spermatozoa was found to vary markedly from sample to sample, the ratio of arginine to DNA per set of chromosomes was four times this ratio for ova and liver nuclei, suggesting a higher proportion of arginine-rich proteins in the spermatozoa than in the nuclei of ova and liver cells. This finding may indicate that spermatozoa and cells of the same species differ in the types of proteins present.

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THE UTILIZATION OF PHOSPHOLIPIDES BY BOVINE SPERMATOZOA

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Histochemical [22, 19] and chemical investigations [4, 8, 18, 24] of the spermatozoon have shown that the sperm cell membrane and other sub-cellular structures are composed in good part of phospholipides and lipoproteins. The loss of phospholipide or lipoprotein from the sperm cell through the action of lecithinases [15] and detergents [7, 15], or in the process of aging [7, 17], has been reported to result in loss of motility, increased susceptibility to thermal shock, and reduced metabolic activity. Conversely, media containing egg yolk, lecithin, or egg-yolk lipoprotein protect spermatozoa from the adverse effects described above.

The loss of phospholipide from the sperm cell is not solely a result of a diffusion process according to Lardy *et al.* [9, 10, 11, 12]. They observed a 50 per cent decrease in lipid phosphorus in semen samples stored 24 hours aerobically at 10°. A concomitant 80 per cent drop in motility was noted. With spermatozoa washed free of seminal plasma and furnished no utilizable substrate similar changes were obtained, indicating a high percentage loss of cell phospholipide. When phospholipide was added to washed spermatozoan suspensions in the absence of a substrate, an average increase in oxygen uptake of 38 and 46 per cent above the endogenous rate for the first and second hours, respectively, was observed. It was concluded by Lardy *et al.* that bovine spermatozoa oxidize cellular or added phospholipides.

The data presented in this study were obtained in an attempt to determine, chiefly, whether blocking of phospholipide oxidation by spermatozoa would prolong the duration of viability. However, the data indicate that bovine spermatozoa, ejaculated or epididymal in origin, do not have the ability to oxidize added or cellular phospholipides to any great extent.

METHODS

Semen was collected by artificial vagina from dairy bulls and kept at room temperature until used unless otherwise stated. Bovine epididymides were obtained from

cal packing house[1] and the spermatozoa flushed through an incision with Krebs-ger phosphate solution. For washing semen was diluted at 1:10 and epididymal rmatozoa at 1:6 with Krebs–Ringer phosphate. The diluted suspensions were n centrifuged at about 2800 r.p.m. in an International centrifuge, head No. 240, 5 minutes. The supernatants were then aspirated. The process was repeated for cessive washings, and the aspirated suspensions were diluted to a concentration about 4×10^8 spermatozoa per ml Calcium-free Krebs–Ringer phosphate solution, 6.9 for ejaculated sperm and 7.4 for epididymal sperm, was the suspending dium in all experiments unless otherwise stated. Motilities were estimated by roscopic observation of samples on a warming stage at 37°. Spermatozoa counts re made with a hemacytometer.

Analytical methods.—Sugar was determined by the method of Roe [23], lactic acid the method of Barker and Summerson [2]. For the determination of lipid phos-rus, spermatozoan suspensions were pipetted into cold 10 per cent trichloroacetic d, let stand 5 minutes at 3°, centrifuged, and the supernatant discarded. The rmatozoa were extracted twice more with trichloroacetic acid in the cold, then racted 3 times with a total of 18 ml boiling 3:1 alcohol–ether solution. The extracts e combined, evaporated, and handled by the method of LePage [14] for total sphorus. Phosphorus was then determined by the method of Fiske and Subba-r [5].

Preparation of lecithin.—Lecithin I was prepared by the method of Pangborn [21], ithin II by the procedure of Hanahan and Jayko [6], Lecithin III was lecithin II litionally purified by the method of Lea *et al.* [13]. Lecithin IV was lecithin III alytically hydrogenated by the method of Hanahan [6] after first reducing the alyst and washing it several times with alcohol acidified with acetic acid. The drogenated product, 1-α-dipalmitoyl lecithin, was then purified by the method Baer [1]. Lecithin V was synthetically prepared 1-α-dimyristoyl lecithin. Lecithin was synthetically prepared 1-α-dioleyllecithin. Both synthetic lecithins were the erous gift of Dr. Erich Baer of the University of Toronto. The purity of the thins isolated from natural sources was checked by the paper chromatographic thod of Lea *et al.* [13]. Lecithins were isolated from egg yolk, rat liver, algae, and mmercial baker's yeast. The C^{14}-labeled lecithins were prepared from yeast in the owing manner. To the sterilized medium of Olson and Johnson [20] were added mc C^{14}carboxyl-labeled acetate and 0.1 mc C^{14}methyl-labeled acetate in sterile ution. Glucose was used at a concentration of 5 g per liter, and asparagine was itted. The medium was then inoculated with a culture of *Saccharomyces cerevisiae* 30 obtained from Dr. Elizabeth McCoy of the Department of Bacteriology, Univer-y of Wisconsin. The yeast was then grown by the method of Olson and Johnson]. The yeast cells were harvested, washed 3 times with about a 50-fold volume water each time, and partially dried *in vacuo*. The extraction procedure of Hanahan l Jayko [6] for yeast lecithin was then used. To the crude alcohol–ether extract out 400 mg of yeast lecithin II was added, the extract evaporated, the residue en up in ether, and the lecithin precipitated with acetone. The precipitate was n used to prepare lecithin III or lecithin IV. To check the radiochemical purity

[1] We are gratefully indebted to Oscar Mayer and Company for their cooperation in securing rine epididymides.

of these preparations, they were chromatographed on paper and developed by the method of Lea *et al.* [13]. The paper strips were cut into about 2 cm pieces and assayed for radioactivity. No radioactivity was found with any preparation other than at the spot corresponding to lecithin. Some C^{14}-labeled lecithin II preparations were made using algae grown in a $C^{14}O_2$ atmosphere or rat livers from rats fed C^{14}-labeled acetate. These preparations were not radiochemically or chemically pure.

Radioactivity measurements.—All samples were assayed for radioactivity in a flowing gas counter, at least 1000 counts being totalled for each sample. Samples were counted at thicknesses of 3 to 10 mg. per sq.cm. and corrected for self-absorption to 1 mg per sq.cm. Carbon dioxide was counted as barium carbonate or sodium carbonate or both. All other samples were counted as previously described [3].

RESULTS

Disappearance of cellular phospholipide.—In this laboratory it has been observed that, occasionally, especially with epididymal spermatozoa, the oxygen uptake obtained with suspensions washed essentially free of seminal plasma exceeds the theoretical value possible from the complete oxidation of the sugar and lactic acid available. To explain the phenomenon confirmation of the use of cellular phospholipides was sought. As shown in Table I (the data are representative of more than 25 experiments with 15 different bulls) when allowed to metabolize endogenously for 1–2 hours at 37.5° in Warburg flasks, washed spermatozoan suspensions with one exception lost little or no phospholipide. The values obtained were the same whether or not sugar was added at zero time. This fact indicates that diffusion, rather than oxidation, was probably responsible for any observed phospholipide losses. In experiments 2, 3, and 8, the actual oxygen uptake far exceeded the values calculated from the complete oxidation of available substrates. It may be noted that the motility of most spermatozoan suspensions dropped markedly during the experiment without concomitant loss of lipid phosphorus. To insure that the washing procedure did not remove a good part of the cellular phospholipide, the washing process was performed individually on semen samples from 6 different bulls. No loss of phospholipide was observed from spermatozoa washed 5 successive times at a 10-fold dilution. Results essentially similar to those observed with washed spermatozoa in Warburg flasks were obtained with semen samples stored at about 10° for 24 hours and also with washed and stored suspensions, as shown in Table I.

Effect on oxygen uptake and motility.—Regardless of the method of preparation of lecithin, the oxygen uptake of washed spermatozoa and their motility response to added lecithin was essentially similar. The data of Table II, which were selected at random from over 100 experiments conducted during

TABLE I

Changes in lipid phosphorus content of sperm.

ot.).	No. of washings	Total motility, per cent Pre-wash	Post-wash	Final	Residual sugar in washed sample μg per ml	Lipid phosphorus content of spermatozoa Micrograms per 10^8 cells Zero time	End experiment
.	1	60	55	40	122	7.1	7.1
2	2	60	55	10	12.2	6.8	5.7(2)
3	2	60	60	10	29.2	7.8	7.6(3)
4	2	60	60	40	102	6.8	6.6(3)
5	2	60	60	10	32	7.6	7.2(3)
6(1)	1	–	50	50	40	8.0	8.0(3)
7(1)	1	–	60	25	22	8.7	8.5(3)
8	2	60	55	40	46	5.4	5.4(3)
9	2	60	50	10	36	6.8	6.8(3)
0	0	65	–	20	–	6.8	6.4
1	0	60	–	30	–	7.2	7.2
2	0	70	–	30	–	7.4	7.3
3	0	65	–	20	–	6.6	6.5

permatozoa for experiments 1–5 and 8, 9 washed at ten-fold dilution with Krebs–Ringer sphate solution at pH 6.9. For experiments 6, 7 cells washed once at 1:6 dilution with pH 7.4 bs–Ringer phosphate. Spermatozoa were incubated for expts. 1–8 in Micro Warburg flasks at ° for 1–2 hours, for expt. 9 at 25° for 9 hours in test tubes, for expts. 10–13 at 10 ± 1° for ours in test tubes. Average concentration for washed samples = 3.5×10^8 spermatozoa per ml.

) Epididymal spermatozoa.
) 5.4 micrograms phosphorus per 10^8 cells with glucose or fructose added.
) Same value obtained with fructose or glucose added at zero time.

ious phases of this study, illustrate the effect of lecithin on washed ejacu-d or epididymal spermatozoa. In every instance the motility of the cells h added lecithin was markedly superior at the end of the experiment to se without added lecithin. The effect was observed with all spermatozoan pensions regardless of the pre-incubation storage time which varied from minutes to six hours. The concentration of the cells in the suspensions d no effect on the maintenance of motility by added lecithin as shown in ble II.

The magnitude of stimulation of the endogenous oxygen uptake by added thin varied considerably with the spermatozoan suspension and the thin preparation. The data of Table II show that different suspensions

TABLE II

Effect of added lecithin on washed spermatozoa

Expt. No.	Pre-use storage time hours	Cell count × 10^8 per flask	Motility, per cent			Oxygen uptake, microliters per flask				Lecithin preparation	Duration of expt. minutes
			Initial	Final		Observed		Calculated (3)			
				Endogenous	Plus lecithin	Endogenous	Plus lecithin	Endogenous	Plus lecithin		
1(1)	5	2.99	60	10	55	105	109	54	38	I	160
2(2)	0.75	4.70	60	30	60	49	57	40	40	I	80
3(2)	0.9	3.10	60	40	50	44	61	37	37	I	110
4(1)	3	4.18	50	30	50	41	51	74	74	I	160
5(2)	2	2.66	60	20	40	53	69	28	28	I	270
6(1)	3	3.00	60	20	40	40	62	50	50	I	160
7(2)	1	2.50	60	30	40	37	60	33	33	II	100
8(1)	3	2.23	60	10	60	33	70	33	33	II	120
9(1)	3	5.2	60	10	50	58	63	35	35	II	140
10(2)	1	4.1	70	30	60	55	89	41	41	II	120
11(1)	4	3.1	65	10	50	82	93	–	–	III	140
12(1)	3.5	3.2	60	20	50	33	43	–	–	III	60
13(1)	3.5	2.5	50	10	40	33	37	–	–	IV	80
14(1)	3.5	2.0	70	20	55	25	40	–	–	IV	80

(1) Ejaculated semen stored at room temperature, then washed twice at 1:10 dilution with Krebs-Ringer phosphate, pH 6.9.

(2) Epididymal spermatozoa washed once at 1:6 dilution with Krebs–Ringer phosphate, pH 7.4. All suspensions were incubated at 37.5° in Warburg flasks with air as gas atmosphere.

(3) Calculated on basis of complete oxidation of sugar and lactic acid utilized.

gave 0 to 40 per cent increases in endogenous oxygen uptake with added lecithin I and 0 to 100 per cent increases with lecithin II. In general, the greatest response was obtained with lecithin II, which contained some lysolecithin and probably some fatty acid impurities. The smallest response was with lecithin I, the least pure of the lecithins used, and which may well have contained inhibitory material among its impurities. Added lysolecithin or palmitate in conjunction with lecithin III, which contained no detectable lysolecithin or fatty acids, stimulated oxygen uptake from 4 to 20 per cent above the uptake observed with lecithin III alone. Thus, impurities in lecithin II account for some of its stimulatory effect.

Whatever the source of the lecithin and its method of preparation, the absolute rate of oxygen uptake in the presence of lecithin was high during the first 40 to 60 minutes of incubation only. Thereafter the rate fell to a level

TABLE III

Effect of lecithin concentration on oxygen uptake.

ot. p.	Lecithin preparation	Oxygen uptake, microliters per flask							
		Endogenous	Lecithin conc. (μg):						
			100	200	500	1000	2000	5000	8000
[1])	II	40.0	44.4	41.2	41.9	44.3	55.8	66.5	–
[2])	V	28.7	–	31.7	33.1	–	42.0	–	–
[1])	III	31.0	–	–	30.4	30.6	–	37.6	–
	V	31.0	–	–	32.5	–	35.2	–	–
[1])	III	35.0	–	–	34.5	40.2	–	45.0	–
	V	35.0	–	–	35.0	–	38.0	–	–
[2])	III	30.0	–	–	29.4	28.4	34.4	35.1	43.3
[2])	III	23.0	–	–	24.0	23.1	28.2	29.5	37.2
[2])	III	22.0	–	–	22.0	20.5	27.1	24.9	53.6
[1])	IV	25.1	–	–	25.8	25.6	36.7	44.1	–
[1])	VI	25.2	26.3	–	26.1	35.4	42.1	45.9	–

) Ejaculated spermatozoa washed twice at 1:10 dilution with Krebs-Ringer phosphate, pH 6.9.
) Epididymal spermatozoa washed once at 1:6 dilution with Krebs-Ringer phosphate, pH 7.4.
)ensions were incubated in Micro Warburg flasks at 37.5° for 80 minutes with air as gas
)sphere.

)roaching that of the endogenous. Attempts to prolong the initial rate of
gen uptake or to increase the oxygen uptake by the addition of possible
actors for the oxidation of lecithin were fruitless.

Effect of lecithin concentration.—If extracellular lecithin is indeed oxidiz-
e by washed spermatozoan suspensions, it would be expected that a
)onse in oxygen uptake would be obtained at any concentration of lecithin
led. Furthermore, a maximum in the rate of oxygen uptake should be
erved once the concentration of added lecithin exceeds the solubility of
thin in the medium. As may be seen from the data given in Table III,
thin failed to stimulate oxygen uptake in all experiments at concentrations
00 μg/ml or lower. In six of the eight experiments in which the 1000 μg/ml
centration was tested, no stimulation occurred. Above 1000 μg/ml in all
eriments, marked stimulation was observed. Thus, the concentration of
thin necessary to effect stimulation of oxygen uptake far exceeded the
)bility of lecithin in aqueous media (the solubility of lecithin III from
yolk was 265 μg/ml). The possibility that substances in the cell membrane
y have to be saturated with lecithin before any lecithin is free for enzy-

TABLE IV

Utilization of C^{14}-labeled lecithin by washed spermatozoa (1).

Oxygen uptake, microliters per flask				Carbon dioxide evolved microliters per flask plus lecithin IV	Added	Total radioactivity, counts per minute of carbon dioxide evolved (4)	
Observed		Calculated (2)					
Endogenous	Plus lecithin IV	Endogenous	Plus lecithin IV			Observed	Calculated (5)
178	266	49.5	49.5	279(3)	14.2×10^4	43	660

(1) Epididymal spermatozoa washed once at 1:6 with Krebs Ringer phosphate, pH 7.0.
(2) Calculated on assumption of complete oxidation of the sugar and lactic acid utilized.
(3) Average of two flasks.
(4) From two flasks.
(5) See text explanation for Table V.

Spermatozoa suspension was incubated with air as gas atmosphere in Warburg flasks at 37.5° for 140 minutes.
Spermatozoan concentration: 10.4×10^8 per flask. Lecithin concentration: 5.07 mg per ml.
Specific activity of lecithin: 4510 counts per min. per mg.

matic reactions was considered. However, it was found that the concentration of lecithin necessary for stimulation of oxygen uptake was independent of spermatozoan concentration.

Utilization of C^{14}-labeled lecithin.—To demonstrate unequivocally the oxidation of lecithin and the extent of the oxidation compared with the overall endogenous oxygen uptake, uniformly C^{14}-labeled lecithin was employed. With lecithin III preparations it was calculated that the lecithin oxidized accounted for 43 per cent of the increase in oxygen uptake caused by the addition of lecithin. The range of values was from 38 to 46 per cent in three separate experiments. With a more highly purified lecithin IV preparation, the calculated value was only 6.6 per cent as derived from the data of Table IV. This figure was obtained in the following manner. First, the observed endogenous oxygen uptake, 178 μl, was subtracted from the observed oxygen uptake, 266 μl, in the presence of lecithin. This difference from Table IV was 86 μl per flask. (Heat-killed controls and flasks with lecithin but no spermatozoa exhibited no oxygen uptake.) From this value the difference, in this example, 0 μl, between the calculated oxygen uptake, 49.5 μl of the lecithin-containing flask and that of the endogenous, 49.5 μl, was subtracted. Thus, 86 μl–0 μl = 86 μl, the excess of oxygen uptake by the lecithin-containing flask over that of the endogenous due to the oxidation of substrates other than sugar or lactic acid. This value, 86 μl, was then multiplied by the number

flasks, 2, giving 172 μl, the total excess oxygen uptake. This value, 172 μl, s converted to micrograms of carbon assuming, as was valid for this periment, that the excess of carbon dioxide evolved was equal to the ess of oxygen consumed. For Table IV:

$$\frac{176\ \mu l \times 12\ \mu g/\mu M}{22.4\ \mu l/\mu M} = 94.5\ \mu g \text{ carbon.}$$

e assumption was then made that the lecithin was uniformly labeled and specific activity of carbon in it calculated. For the data of Table IV:

$$\frac{4510\ \text{c/m/mg lecithin}}{643\ \mu g\ \text{carbon/mg}} = 7.0\ \text{c/m}/\mu g \text{ carbon.}$$

en the total count of the evolved carbon dioxide should have been 94.5 μg bon $\times$ 7.0 c/m/μg = 661 c/m.

DISCUSSION

Use of cellular phospholipides.—The negative results obtained in testing ability of washed bovine spermatozoa to oxidize cellular phospholipides flatly contradictory to previous reports. One possible explanation is that spermatozoa in the present study may have been more thoroughly washed n those employed in earlier studies. It is known [16] that seminal plasma tains phosphatases. One or more of these phosphatases may hydrolyze minal acetal phosphatides or spermatozoan cellular phosphatides. The drolysis products may then be oxidizable by the cells. It is believed that far lower endogenous oxygen uptake values and shorter periods of tility observed in this work compared to those reported in previous work indicative of more complete removal of seminal plasma by our washing cedure than by earlier methods. In support of the above possibility, it s found that semen washed only once and then incubated at 37.5°C for riods of 3 to 4 hours did lose phospholipide. In 5 separate experiments, 9, 12, 20, and 35 per cent of the phospholipide disappeared from the ubated suspensions. Some of the lost phospholipide was cellular phos-olipide and some plasma phospholipide, but the quantities lost from each rce were not measured. The fact that no disappearance of cellular phos-olipide was observed with epididymal spermatozoa lends further support the above discussion.

Use of added lecithin.—The data presented indicate that washed bovine rmatozoa are unable to oxidize added lecithin. Probably, if a radio-

chemically pure uniformly labeled lecithin were employed, no radioactivity would be observed in the evolved CO_2. This is indicated by the fact that with lecithin III preparations, the average observed radioactivity of evolved CO_2 in three separate experiments was 43 per cent of that calculated. With the lecithin IV preparation, the observed value was only 6.7 per cent of that calculated. With lecithin II preparations the observed value was equal to or higher than that calculated. As stated previously, impurities were found in lecithin II, but not in lecithin III or IV. However, with lecithin III preparations, an initial period of inhibition of oxygen uptake was observed quite frequently. With lecithin IV or with synthetically prepared lecithins, no inhibitory period was noted. Therefore, radioactive lecithin III preparations may well have contained a trace amount of oxidizable radioactive impurity which was absent or present in smaller quantity in lecithin IV. In all experiments with radioactive lecithin the radioactivity of the evolved CO_2 was less than 0.1 per cent of the radioactivity added.

Mechanism of action of lecithin.—It is well known that phospholipides affect spermatozoa other than by acting as an energy source. Thus, energy-providing substrates will not protect ejaculated spermatozoa against the adverse effects of thermal shock while phospholipides and phospholipide complexes will. Spermatozoa that have undergone thermal shock lose a small part of their membrane phospholipide and exhibit altered permeability characteristics [17]. More immediate changes, however, are almost complete loss of motility and a large decrease in the adenosine triphosphate content. It was found in this laboratory that spermatozoan suspensions to which lecithin was added after shock treatment failed to regain their pre-shock motility. Similar to the effects of thermal shock, detergent treatment of spermatozoa results in altered membrane properties, and loss of motility, phospholipides, and metabolic activity [7]. The detergent effects can be partially reversed by the addition of lecithin if detergent action has not proceeded too far, as observed by Lovelock [15] and in this laboratory. However, the present authors have found that, if lecithin was added to the sperm suspension before the detergent, no adverse effects on spermatozoan motility or metabolic capacity were observed in the presence of an oxidizable susbtrate. With lecithin added before the addition of the detergent, exposure to the detergent was over a much longer period of time and at higher temperatures than the exposure required to effect changes irreversible by lecithin. The presence of an oxidizable substrate without added lecithin did not prevent nor reverse the detergent effects. Thus, whatever the beneficial action of lecithin on spermatozoa, it probably occurs immediately on addition to

rmatozoan suspensions. It appears most probably that lecithin, by chemi- or physical action, renders essential membrane components less subject adverse physical and/or chemical conditions. With "normal" intact mbranes the spermatozoa would not lose co-factors necessary for metabo- n as they do [7] under the influence of detergents or in the process of ng. The reason for the observed stimulation of endogenous oxygen uptake added lecithin then would be the prevention by lecithin of diffusion from cell of co-factors necessary for metabolism. In support of this hypothesis he action of lecithin on beta-hydroxybutyrate oxidation as observed in laboratory. Beta-hydroxybutyrate supported respiration of washed sper- tozoa at 30° for periods of about one hour only. Thereafter, the respira- n rate rapidly fell toward the endogenous rate. The addition of adenosine nophosphate, adenosine diphosphate, or lecithin with beta-hydroxybu- ate maintained the respiration rate at the maximum value observed with a-hydroxybutyrate alone for periods of about two hours.

The authors hope to report on the mechanism of lecithin action and the ure of the spermatozoan endogenous substrate in the near future.

SUMMARY

. Ejaculated spermatozoa, washed essentially free of seminal plasma, d washed epididymal spermatozoa lost little or no cellular phospholipides en aerobically incubated without added substrate.

. Lecithin added to washed spermatozoa stimulated endogenous oxygen ake and prolonged motility of the cells. A portion of the observed oxygen rease was probably from oxidation of impurities in some lecithin prepara- ns.

. The concentration of lecithin necessary to effect maximum stimulation oxygen uptake far exceeded the solubility of lecithin.

. The "oxidation" of "pure" C^{14}-labeled lecithin by washed epididymal rmatozoa was only 6.6 per cent of the increased oxidation caused by the lition of lecithin.

. The data presented indicate that bovine spermatozoa do not have the lity to oxidize phospholipides to any great extent.

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MORPHOLOGY AND FINE STRUCTURE OF THE SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

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E genus *Dictyostelium* within the Acrasiales was first recognized in 1869 Brefeld, who described *D. mucoroides*. The genus has been the subject thorough investigations because of its interesting life cycle. These in-igations have been intensified since 1935, when *D. discoideum* was ated by Raper.

he development of a *Dictyostelium* species starts with a vegetative stage unicellular, free-living myxamoebae feeding on bacteria. According to son [31] this stage ends by the conversion of the vegetative cells into hetic amoebae, which fuse to zygotes. The diploid cells formed first act centers for other zygotes, which aggregate to pseudoplasmodia. In the udoplasmodium meiosis immediately starts and successively takes place ughout the whole cell collective [31]. Aggregation being finished, the udoplasmodium differentiates during culmination to the mature fruiting e or sorocarp, which consists of sorus, stalk or sorophore and, in *D. oideum*, a basal disk.

. discoideum differs from the other species by a delayed sorocarp forma-. The pseudoplasmodium does not culminate directly after aggregation migrates as a unit (a densely packed group) over the substratum for indefinite length of time. (Quite recently another migrating species, *olycephalum*, has been isolated [19]).

he sorocarp—a very complex structure for such a primitive organism as *tyostelium*—and its formation has been described by Raper and Fennell]. The sorophore is surrounded by a continuous, hyaline sheath, which laimed to consist of cellulose. The sheath is first formed as a very thin l fragile tube during early culmination, and it elongates and thickens until ocarp formation is ended. It is interesting to note that the sheath is depo-d extracellularly between the cells in the apical papilla. Extracellular

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cellulose is never found within a cell aggregate; it is only known to be formed by some of the vinegar bacteria, e.g. *Acetobacter xylinum.*

Raper's conclusion that the sheath (and the walls of spores and stalk cells as well) consist of cellulose, is based on some staining reactions typical for cellulose, and on the solubility of the substance in 72 per cent sulphuric acid and in Schweitzer's solution [20].

Furthermore, the substance shows birefringence and is decomposed by cellulolytic bacteria [20].

Preliminary tests on intact sorophores with paper chromatography show the presence of glucose, and X-ray analysis of the intact sorophores gives a very weak diffraction pattern in which only two reflections were identified. The spacings (4.02 and 4.41 Å) agree with those of hydrate cellulose [20].

It is of great theoretical interest to know if the resistant *Dictyostelium* substance, which is deposited extracellularly, is really cellulose. For this reason this substance has been analyzed using a number of physio-chemical methods. In connection with this investigation an attempt has also been made to study in greater detail the morphology of *Dictyostelium* using ultrathin sectioning in combination with electron microscopy. All these investigations were performed at the Institute of Physical Chemistry, University of Uppsala.

Organisms and Cultivation

Lyophilized cultures of *Dictyostelium discoideum V-12* and *Escherichia coli 107* were supplied by Dr. K. B. Raper, N.R.R.L. Peoria, Illinois, U.S.A. The organisms were recultivated according to Dr. Raper's instructions. Since *Dictyostelium* does not grow in one-membered culture without a specific bacterial fraction [29], the cultivation was made in the usual way in streak cultures with *E. coli* as described by Raper [18]. The substratum was peptone–dextrose agar with 1–1.2 per cent of each nutrient. The plates were incubated at 20°C.

All micro-biological work including the optical microscopy was carried out at the Institute of Physiological Botany, University of Uppsala.

Morphological Investigations

Experimental.—The morphological investigations of *Dictyostelium discoideum* were made with optical and electron microscopy.

For optical microscopy both mature sorocarps and pseudoplasmodia in different stages of development were isolated. The fixation was made according to Bonner [5] using a solution containing 3 parts of saturated $HgCl_2$ in water, 2 parts of glacial acetic acid and 5 parts of 96 per cent alcohol. The treated specimens were embedded in paraffin. The sections were obtained with a sliding microtome (thickness 6 μ) and were dyed with Heidenhain's haematoxylin and light green. Sections of soro-

res, sori and culminating pseudoplasmodia were exposed in a Leitz photomicro-
e. The pictures obtained were used for surveying purposes in the subsequent
tron microscopical investigations.

or electron microscopy the specimens were stabilized and stained with osmium
oxide and embedded in polymerizing methacrylates (cf. [14]).

he fixation solution, buffered to a pH of 7.42, was made up from 5 ml 1/7 *M*
acetate–1/7 *M* Na veronal solution, 2 ml 8.5 per cent NaCl, 5 ml 0.1 *M* HCl, 15 ml
. water and 0.25 g OsO_4, which means that its OsO_4 content was 0.9 per cent.
or the fixation the specimens were picked from the agar surface with a very
. pair of stainless steel tweezers and transferred into a weighing glass containing
tion solution. To avoid mechanical damage the specimens were transferred to
fixation solution on a small piece of the supporting agar which was easily separated
he solution. The fixation time was usually 4 hours. For some younger sorocarps,
ll pseudoplasmodia and culminating stages the time was reduced to ¾–2 hours
void brittleness and disintegration during the embedding operation.

he embedding was made mainly according to methods developed by Newman, Bo-
o and Swerdlow [11, 12]. A monomer mixture of butyl and methyl methacrylate,
purified as described by Newman *et al.* [12] in the proportions 4:1 were used with
r cent by weight of benzoylperoxide added as an initiator of the polymerization
tion. The embedding was made in gelatine capsules size No. 1 and the poly-
ization was carried out at ca 50°C in an oven. As the specimens were very small,
y could not be oriented in any specified direction in the capsules.

he fixation of the vegetative amoebae was made according to the method
h-Andersen, Maaløe and Sjöstrand [4] had worked out for *Escherichia coli*.
tyostelium discoideum was grown in streak culture on agar in the usual way. The
elopment of the amoebae was followed using an optical microscope. When the
r surface contained large amounts of amoebae but before aggregation had begun,
incubation was stopped. The amoebae were then still in the vegetative stage of
elopment [31]. The cells were rinsed away from the agar surface using dist.
er, and the agar was then washed again thoroughly with water using a bent
s rod.

o 30 ml of the resulting cell suspension 3 ml of a 2 per cent OsO_4 solution in phos-
te buffer of pH 7.3 was added (the buffer contained 1.45 g KH_2PO_4, 7.60 g Na_2HPO_4.
O and 4.80 g NaCl per litre). The suspension was immediately centrifuged for
minutes. To the residue (which mainly consisted of amoebae due to the weak
trifugation), 2 ml of phosphate buffer and 2 ml of 2 per cent OsO_4 in buffer solution
added. The suspension was incubated for 2 hours at 20°C. After fixation the cells
e washed by centrifugation and then embedded in polymer in the usual way.

he sectioning of the polymerized specimens was performed with a microtome
structed in the Institute of Physical Chemistry, Uppsala [6]. The polymer piece
cut with a razor-blade in such a way that the part of the specimen under investiga-
was in the tip of a pyramid with the base ca. 0.3×0.3 sq.mm. The sections had
average thickness of ca. 300 Å. They were taken up from the water surface directly
100–200 mesh screens covered with formvar membranes and studied in the
tron microscope without removal of the polymer.

he electron microscope used was of Siemens make (Type 1939, ÜM 100) operating
setting of 100 kV and giving a magnification of 8500 diameters (± 2 per cent).

RESULTS

The *vegetative myxamoebae* have no other boundary toward the environment than the plasma membrane and some diffuse substance surrounding the cell—perhaps mucus or slime. The plasma contains vacuoles of varying size and shape, the largest of which is the contractile vacuole in the end of the amoeba. It measures 3–4 μ in diameter. The contraction seems to be effectuated by a ring structure, which does not form the border of the vacuole but is situated within the cytoplasm (Fig. 1).

In the *migrating pseudoplasmodium* the cells have no persistent shape. They fit to each other to make up a tissue-like cell collective. The cells are not separated through any intercellular substance but only by their plasma membranes. The collective is protected by a slime envelope faintly visible as a diffuse substance in Fig. 2. (The slime envelope is obviously dissolved during fixation causing brittleness of the objects.) Within the uninuclear cells there are plenty of easily distinguishable particles staining with OsO_4. Their character is not established but comparison with electron micrographs of different animal cells and some micrographs of cytologically stained preparations of *Allomyces* and *Mycobacterium* in literature indicated that at least some of the particles are mitochondria, while others are lipid inclusions. Because of the fat content of these organelles they appear dark after fixation with OsO_4.

In many cells there are one or two nucleoli, often polarly situated in the nucleus. They reduce OsO_4, though not homogeneously, showing a clearly spotted pattern (Fig. 2). In other cells there are dark bodies, which seem to be located just at the nuclear membrane. Their nature is still in doubt (Fig. 3).

During *culmination* the cells are more differentiated. The pre-spores become more or less rounded in shape and the cells of the sporogeneous mass are thus separated from each other. Different stages of maturing spores with quite different organization of the cytoplasm are seen (Figs. 4–6). No mitoses have been found, possibly because OsO_4 has no affinity to nucleic acids. Formation of the cell wall *(probably double)* begins in the latest stage (Fig. 6).

Sorocarp.—The sorus is not surrounded by any membrane (Fig. 7), the spores being held together in a drop through surface tension. Until complete maturation of the sorocarp, the sorus ends in the apical papilla, where stalk formation takes place. According to Raper and Fennell [20] this papilla is surrounded by a slime envelope throughout the entire development.

The spores reveal the same plasmatic structure as the maturing pre-spores (Fig. 8). No nuclei are seen in this stage because of the cutting direction. Other micrographs, which are not published here because of their technical inferiority, show one nucleus situated medianously in the spore just at the cell membrane. According to Wilson [31] each spore has one or incidentally two nuclei. The latter is the case when there is a lag in cytoplasmatic division after mitosis preceding spore formation. On electron micrographs two nuclei have occasionally been recognized in late stages of maturing pre-spores. The cytoplasm contains numerous organelles with a lipoid membrane like the nucleus. Since this is the case with mitochondria, too, the organelles observed may belong to this kind of cell bodies.

The spores are surrounded by thick, probably double walls which, as previously mentioned, are supposed to contain cellulose. The walls lie tightly to the cell membrane in living spores.

The sorophore consists of a central cell column and a sheath (Figs. 9–10). The cells of the lower two thirds of the column are strongly vacuolated (Fig. 11). The cell walls are connected to each other through intercellular substance. In view of the fact that the cells of the basal disk have the same properties, it is impossible to distinguish stalk cells from basal disk cells (Figs. 12–14).

In the apical papilla the sheath is deposited between the pre-stalk cells and the newly formed unoriented stalk cells [20]. The pre-stalk cells show horizontal to diagonal orientation; their cytoplasm contains vacuoles but resembles that of the migrating stage. The shape of the cells depends on the surrounding cells. The new stalk cells have essentially the same characteristics as the pre-stalk ones. No cell walls are yet formed and no orientation has been in place in the stalk (Figs. 15–16).

At this stage the sheath merely consists of a delicate membrane hardly visible between the cells and not adhering to them (Fig. 16). During culmination it thickens and elongates as described by Raper and Fennell [20] and in the region below the sorus there are two or possibly three layers in the sheath (Fig. 10).

The outmost part of the sheath seems to contain two fibrous layers more or less separated from each other. Through the action of the fixative this part seems to swell like the spore walls and is separated from the inner section. This is much thinner and is connected with the underlying cell walls through intercellular substance.

In the basal disk the sheath is comparably thick (ca. 1 μ, Figs. 13–14) and can, even in the sections, be seen to consist of parallel fibers. The

different parts are indistinguishable. The bulk of the sheath is connected with cell walls on both sides (stalk cells and basal disk cells).

Isolation of the Resistant Framework Substance from Dictyostelium

The material was obtained by growing *Dictyostelium discoideum* in streak cultures on peptone–dextrose agar at 20°C together with *Escherichia coli*. The Petri dishes were incubated for 9–10 days, for maximum growth. The sorocarps were harvested by flushing with dist. water. They were killed by 70 per cent alcohol and were stored in absolute alcohol. The spores were unintentionally separated from the sorophores during this treatment and for this reason their cell wall substance is not included in the specimens isolated here.

The sorophores with their basal disks were washed with dist. water (to remove the alcohol) and then treated on a boiling water bath with 1 per cent caustic soda under nitrogen atmosphere for 20 hours. The caustic solution was changed after 5 and 15 hours [23]. The caustic soda was drained off and the residue was dialyzed against dist. water for 3–4 days to remove electrolytes and low molecular weight material. The purified substance was treated successively with alcohol, acetone and cyclohexane and then dried.

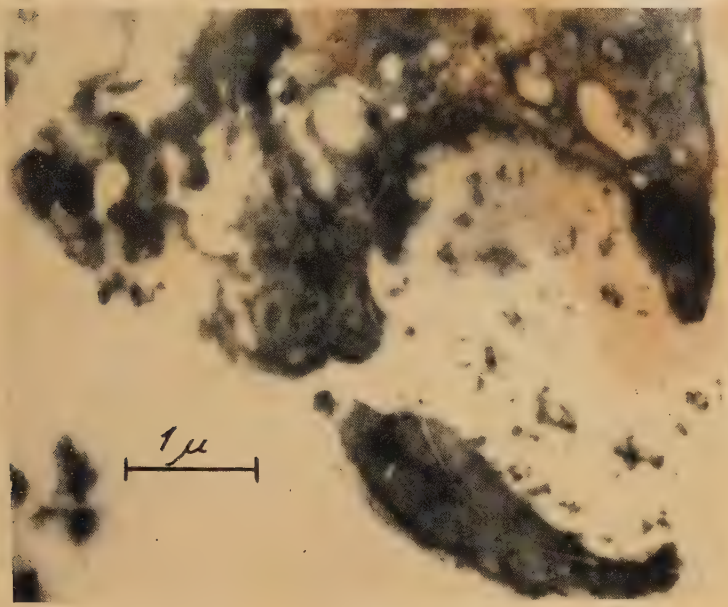
Two preparations were made (Dic 1 and Dic 2). The amount of Dic 1 was not more than 0.02 g. To obtain larger amounts for a more thorough investigation, the mold was grown at a larger scale using 500 Petri dishes of 10 cm diameter and 300 dishes of 15 cm. The total yield of Dic 2 was 0.18 g of purified dried substance.

The sample Dic 2 contained 0.3 per cent nitrogen (acc. to Kjeldahl) which would correspond to approximately 2 per cent residual protein.

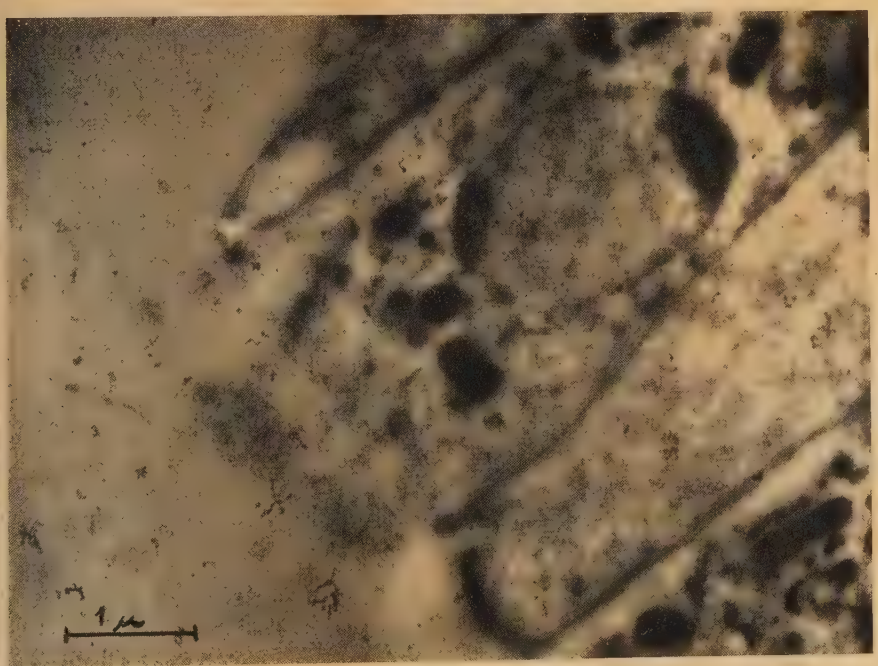
Investigations of the Framework Substance

Paper chromatography.—The composition of the resistant polysaccharide sample Dic 2 was studied. Two samples (0.03 and 0.07 g) were hydrolyzed to monosaccharides according to Saeman *et al.* [28] with 71 per cent sulphuric acid for 1 hour at 30°C, and the hydrolysis was completed at 1–1.2 atm pressure for 1 hour after dilution with water to 2.5 per cent. Whatman paper No. 1 was used for the chromatograms. They were developed with a mixture of ethyl acetate, acetic acid and water in the proportions 3:1:3 [8]. The chromatograms showed only one monosaccharide moving with the same velocity as glucose. To find out if galactose was present, two chromatograms were developed using ethyl acetate–pyridine–water in the proportions 2:1:2 [8] which would separate galactose from glucose. These experiments showed no trace of galactose, and it was concluded that glucose was the only monosaccharide present.

X-ray diffraction.—The X-ray diffraction analysis was performed with a Guinier powder camera, built by Prof. G. Hägg, Institute of Chemistry



1. Posterior part of vegetative myxamoeba showing contractile vacuole, 2 h. fixation, nification, ×17,000. (Electron micrograph, as Figs. 2–6, 8, 10, 11, 13–21.)



2. Cells from the outer part of migrating pseudoplasmodium, 4 h. fixation, ×17,000.

Fig. 3. Cells from migrating pseudoplasmodium, 2 h. fixation, × 17,000.

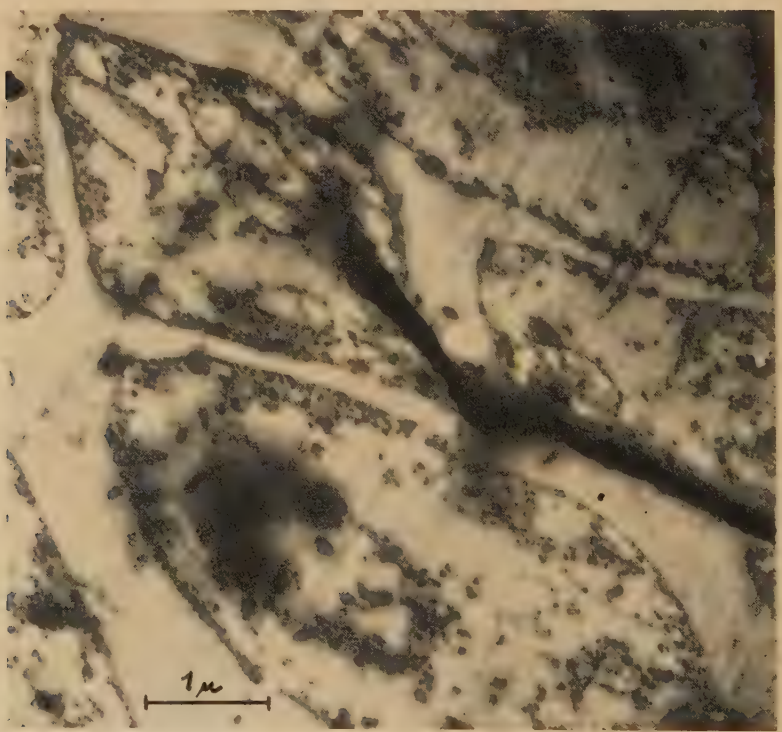
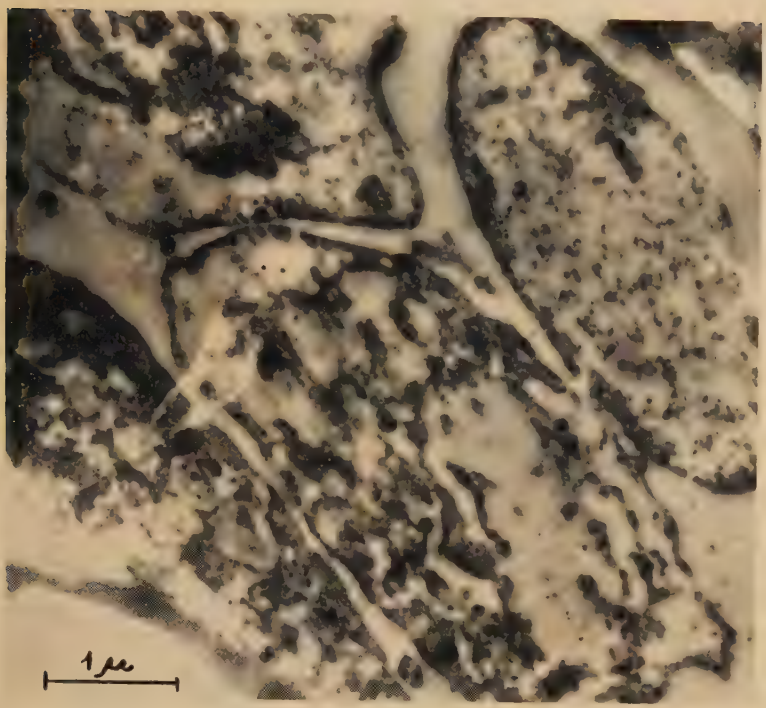
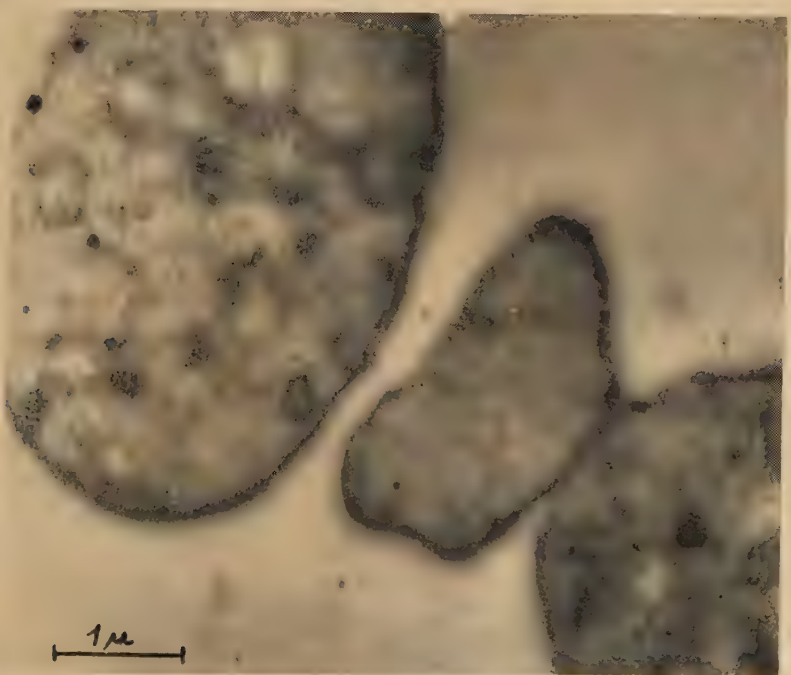


Fig. 4. Pre-spores from young culminating pseudoplasmodium, 4 h. fixation, × 17,000. (The dark band is a fold in the section.)



5. Older pre-spores. 2 h. fixation. 17,000.



6. Maturing pre-spores. Late stage of culmination. 2 h. fixation, × 17,000.

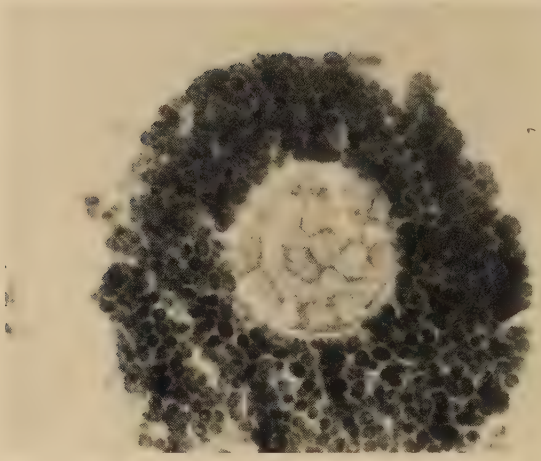


Fig. 7. Cross section through sorus
6μ. Haematoxylin–light green, approx
×540. (Optical micrograph, as Fig
9 and 12.)

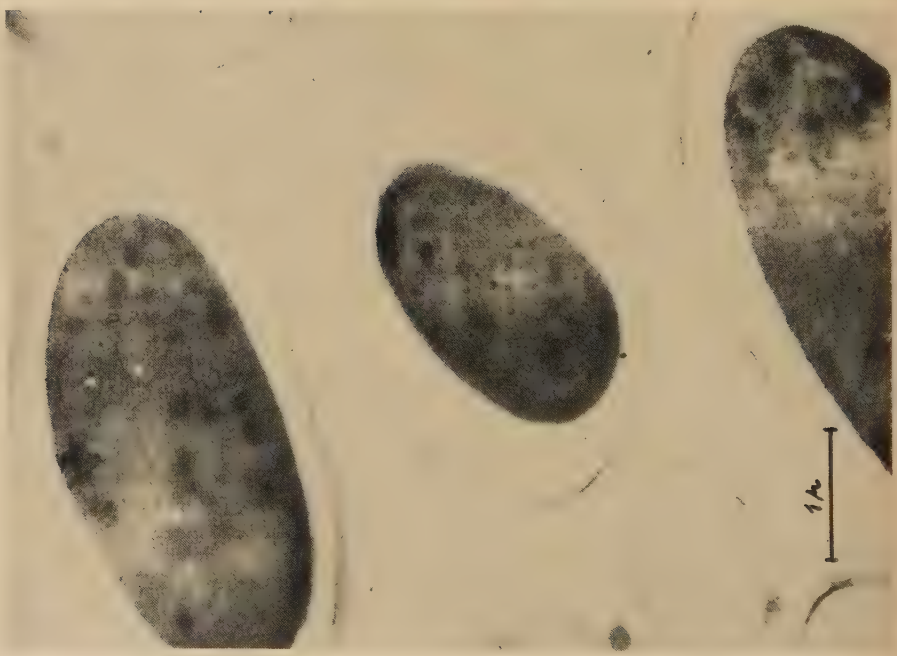
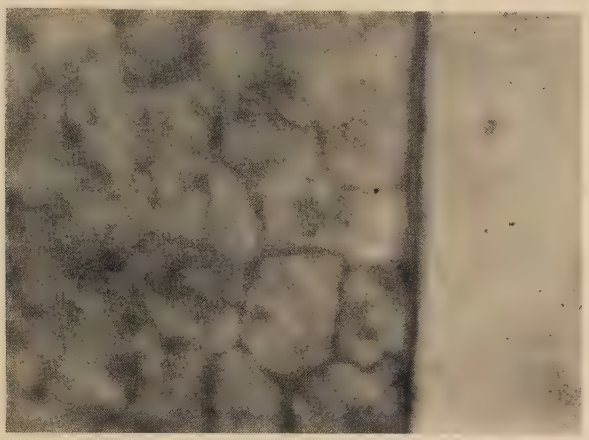
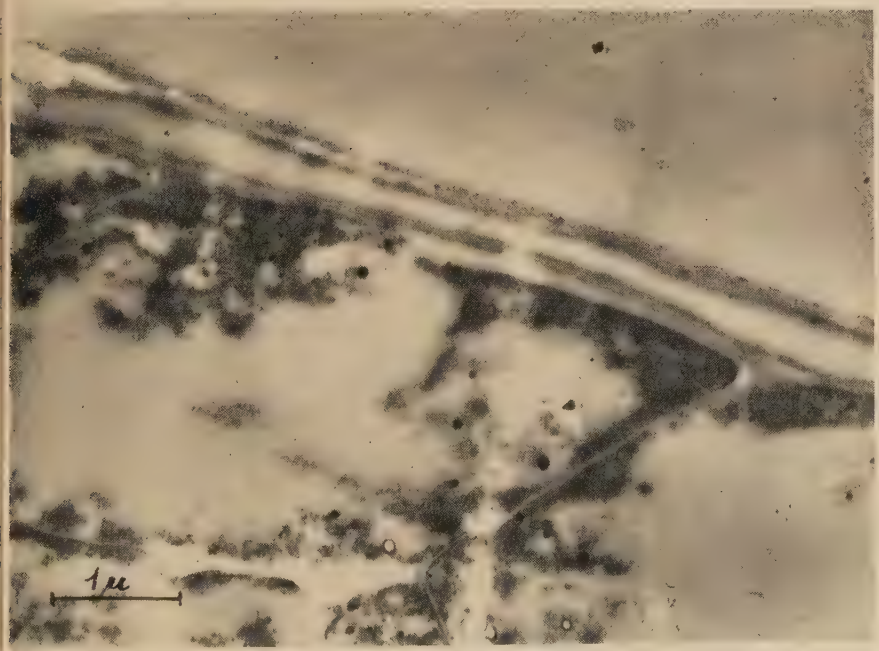


Fig. 8. Spores, 4 h. fixation, ×17,000.



9 Longitudinal section through
phore. 6μ. Haematoxylin–light
n, approx. × 1800.



10. Sorophore sheath and cells below the sorus. 4 h. fixation, × 17,000.

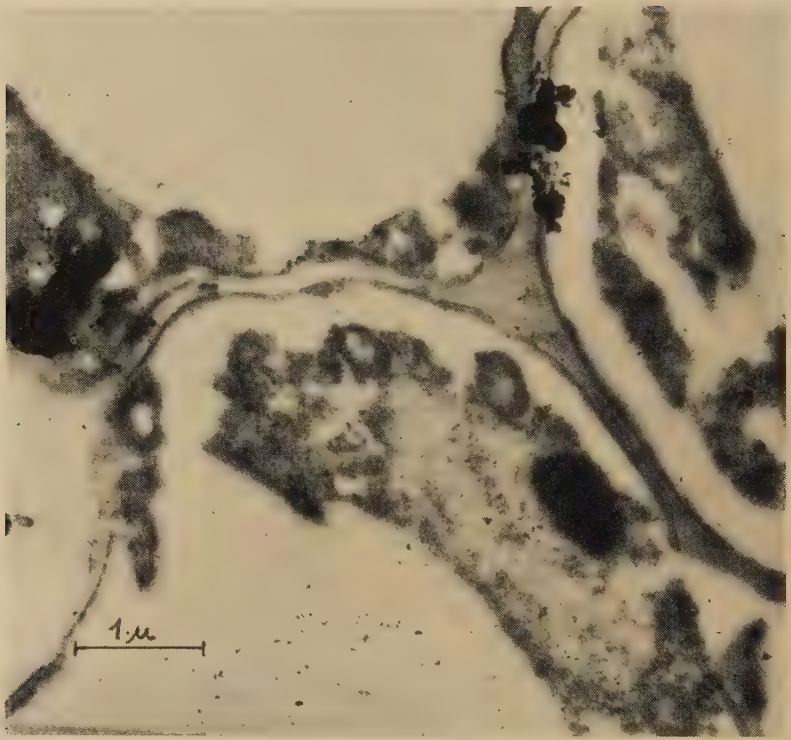


Fig. 11. Vacuolated cells from medium part of sorophore. 4 h. fixation, × 17,000.

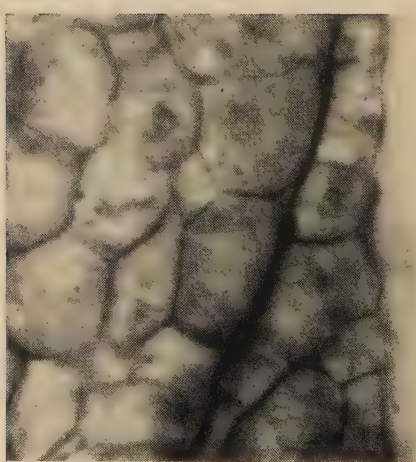
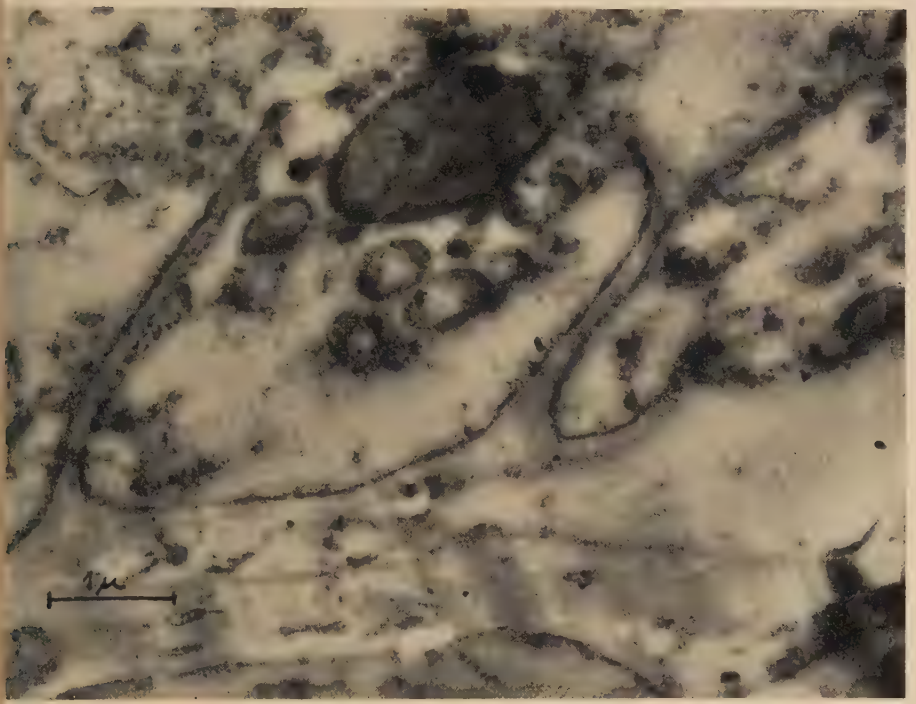
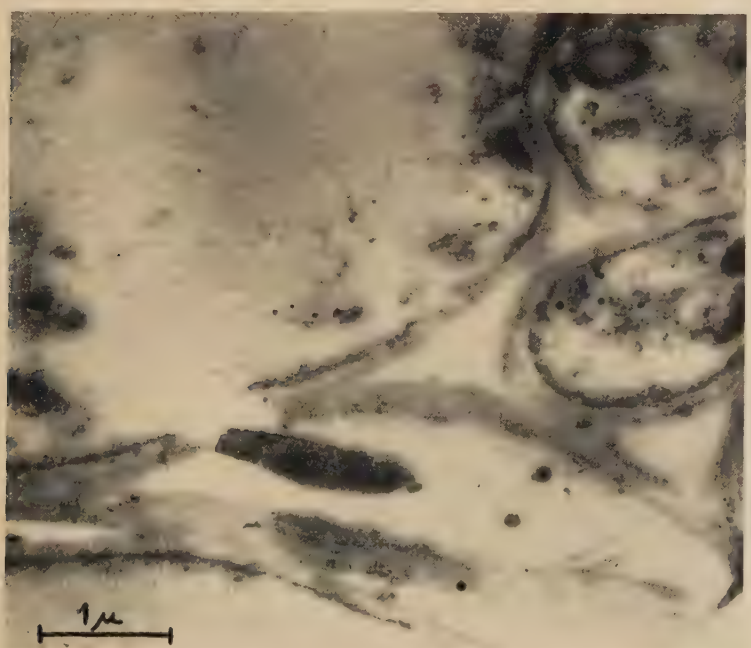


Fig. 12. Longitudinal section 6 μ, through sorophore and basa disk. Haematoxylin–light gree approx. × 1800.



. 13. Sorophore sheath and cells in the basal disk. 4 h. fixation, × 17,000.



. 14. Sorophore sheath and cells in the basal disk. Exposed on the opposite side of the sheath Fig. 13. 4 h. fixation, × 17,000.

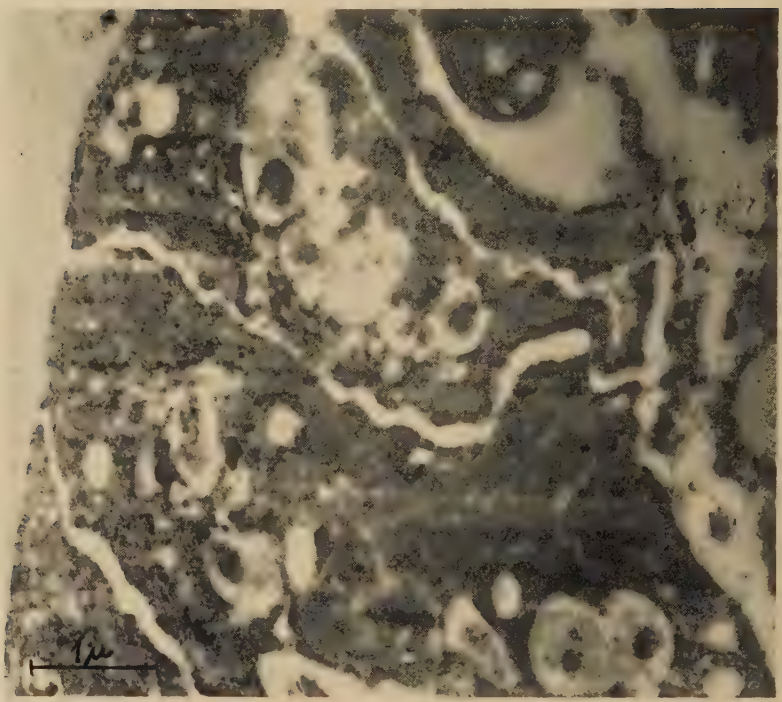


Fig. 15. Pre-stalk cells from apical papilla of sorocarp. 45 min. fixation, × 17,000.

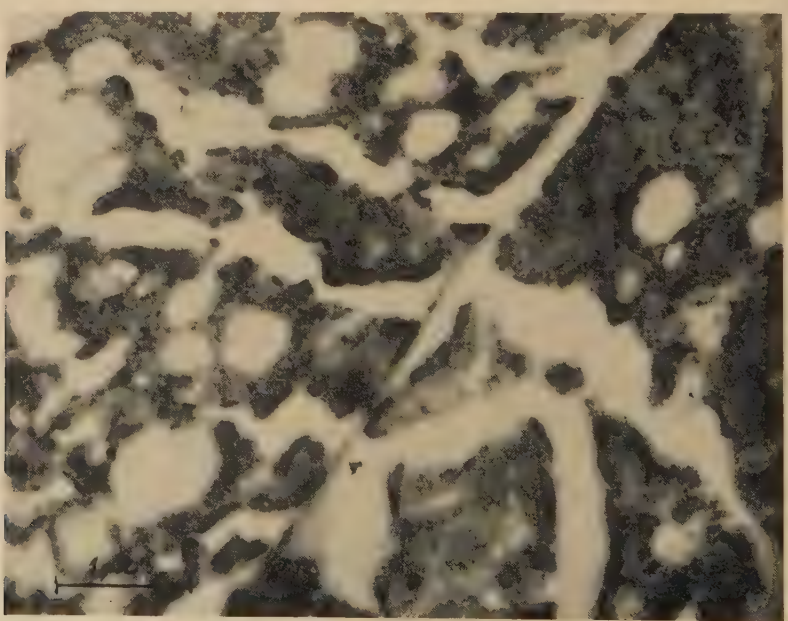
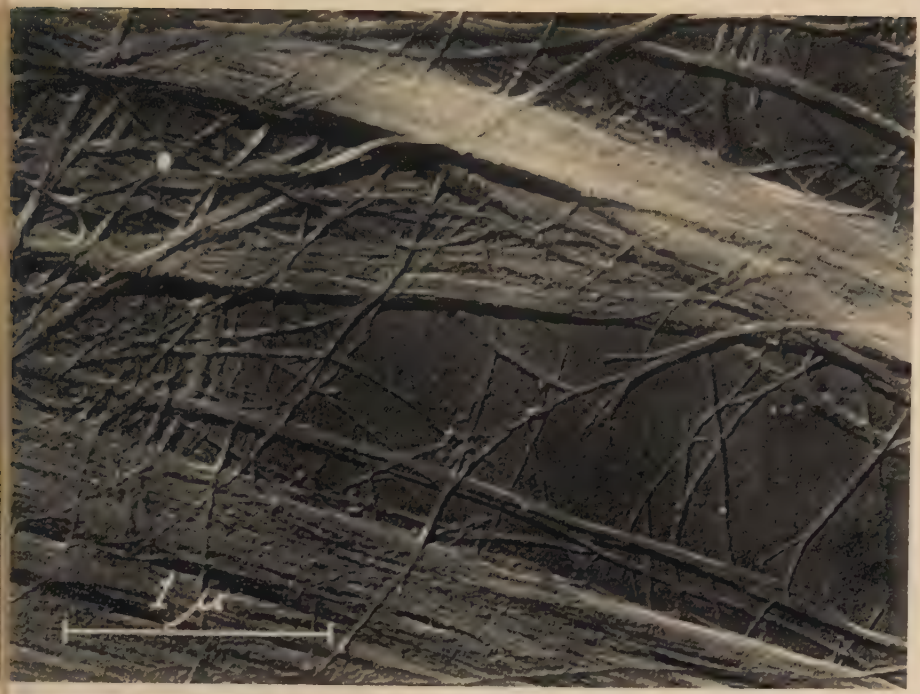
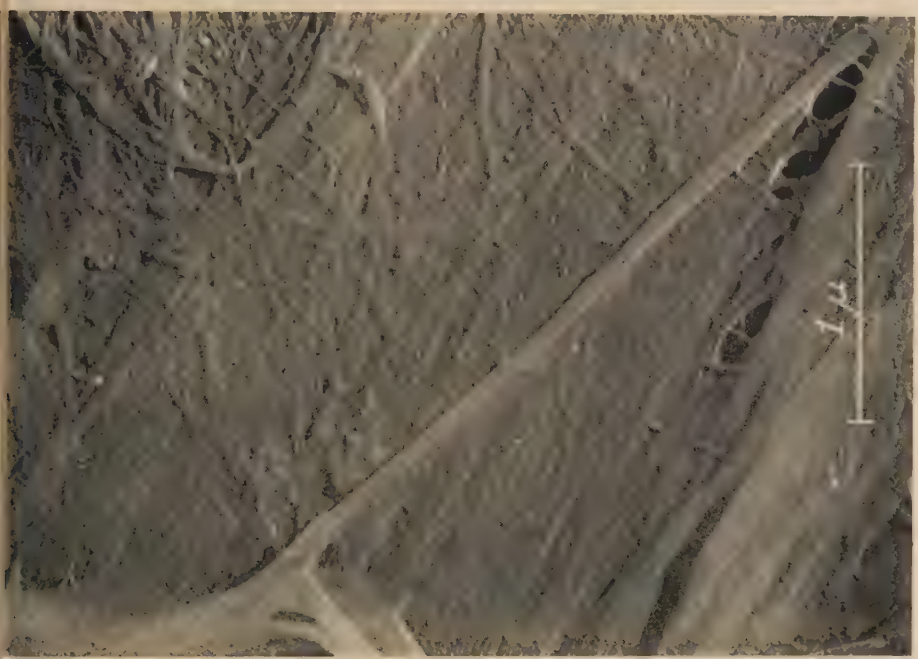


Fig. 16. Newly formed sheath in the apical papilla. 45 min. fixation, × 17,000.





17 and 18. Cellulose from sorophore sheath. Dispersed with ultrasonic irradiation. Au–Mn
owed. × 34,000.

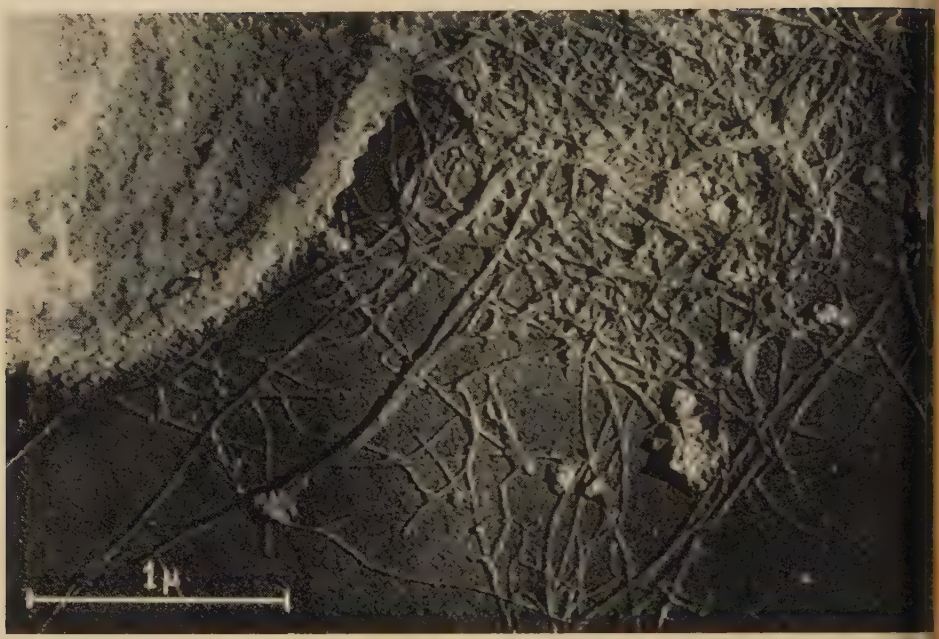


Fig. 19. Dispersed cellulose from stalk cells and sorophore sheath. Au–Mn shadowed. · 34,000.

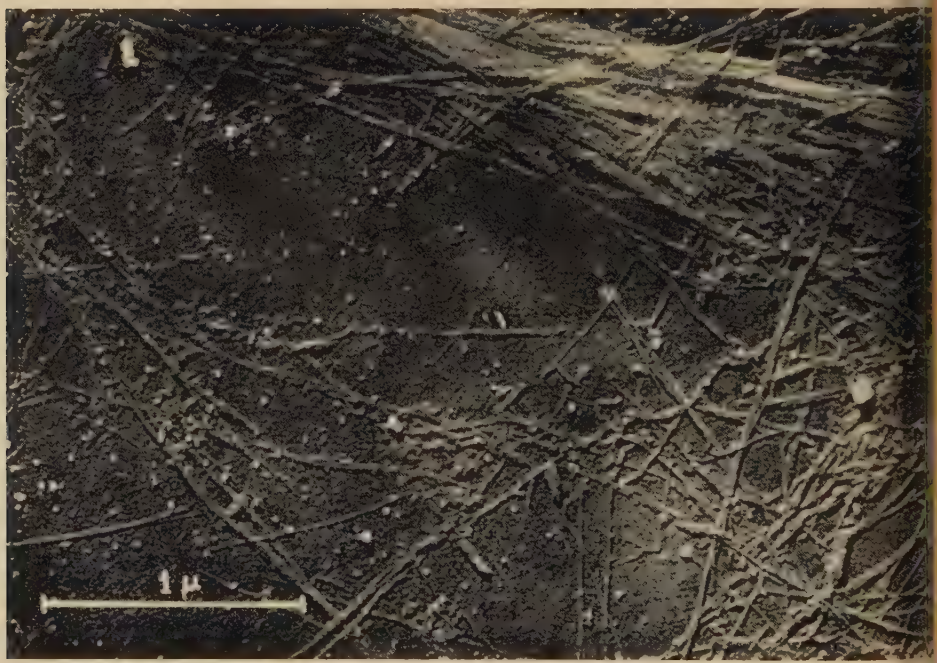
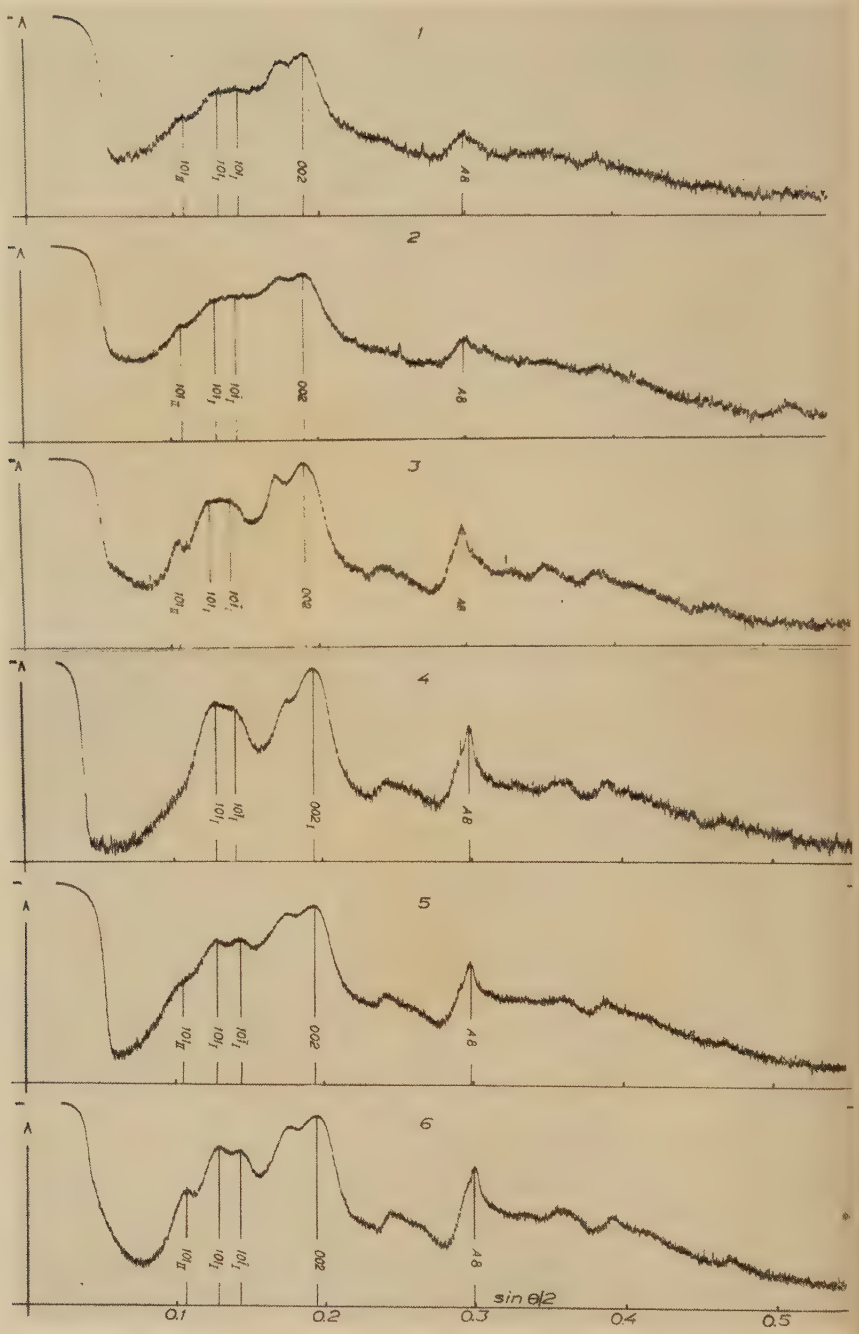


Fig. 20. Dispersed hydrolyzed *Dictyostelium* cellulose. Au–Mn shadowed. × 34,000.

21. (For explanation see Fig. 20.)

versity of Uppsala. The camera used Cu Kα radiation (λ = 1.5387 Å) le monochromatic and focused with a bent quartz crystal. The specimens e mounted in a frame as a self-supporting plate of 1 mm thickness. All ples were exposed for 2 hours and the X-ray films (Ilford) were developed 5 min. in a standard developer (Kodak X-ray). Specimens of the two ated *Dictyostelium* samples Dic 1 and Dic 2 were exposed. They gave tical X-ray diffraction patterns as shown in the photometer curves of


1
2
3
4
5
6
II 101
I 101
I 101
002
AB
I 101
I 101
I 002
sin θ/2
0.1
0.2
0.3
0.4
0.5

films (Diagrams 1 and 2 in Text-fig. 1). The diagrams contain reflexions
$_{I}$, 002_{I} and A8) which are typical for native cellulose lattice (Cellulose I,
p. Table I), but the reflexions are wider and less well resolved than those
native wood cellulose (No. 4 in Text-fig. 1, bleached and alkali-extracted
hite pulp). This means that the *Dictyostelium* substance probably is
ulose of a low lattice order.

a cellulose specimen of low lattice order, e.g. a mercerized or a regen-
ed cellulose, is hydrolyzed heterogeneously using a dilute strong acid,
-ray reflexions become sharper and the background scattering decreases.
s is interpreted to mean both a removal of amorphous cellulose and a
ultaneous recrystallization (an improved lattice order) of the depoly-
ized residue. 0.0490 g of the sample Dic 2 was hydrolyzed for 2 hours
oiling 2.5 *N* sulphuric acid. The residue (Dic H) was only 0.0132 g
per cent yield) after washing and drying. The low yield after hydrolysis
indicates a low crystalline order.

he hydrolyzed substance (0.012 g of Dic H) gave considerably sharper
ay reflexions (No. 3, Text-fig. 1) than the original samples. Beside the
n reflexions, which are characteristic for the native cellulose lattice, a
k reflexion (sin $\theta/2 = 0.107$) appears at the same position as the 101_{II}
nercerized cellulose lattice (Cellulose II). The same reflexion, but much
ker, is also visible in the diagrams of the original samples (Table I).
or comparison the X-ray diffraction pattern of a partly mercerized wood
ulose (a sulphite pulp treated with 7.10 per cent caustic soda at 0°C,
ple No. 15, described by Rånby and Mark [27]) is shown as diagram 5
Text-fig. 1. Sample No. 15 was hydrolyzed in the same way as Dic 2
a yield of 63 per cent (Sample 15 H), which means that the partly
cerized wood cellulose sample is much more resistant to heterogeneous
rolysis than the *Dictyostelium* substance. Its X-ray diffraction pattern
. 6 in Text-fig. 1) contains sharper and better resolved reflexions than
original sample.

comparison of the photometer curves (Text-fig. 1) shows that the ad-
nal reflexion in the *Dictyostelium* pattern most probably is identical with
101_{II}-reflexion of mercerized cellulose (Table I). It can therefore be
cluded that the isolated *Dictyostelium* substance, which according to the
matographic analysis contains only glucose, is most probably a partly

-fig. 1. Photometer curves of X-ray diffraction patterns for (1) *Dictyostelium* cellulose, first
tion, Dic 1; (2) *Dictyostelium* cellulose, second isolation, Dic 2; (3) hydrolyzed *Dictyostelium*
lose, Dic H; (4) native wood cellulose (B 2); (5) partly mercerized wood cellulose (15); (6)
olyzed, partly mercerized wood cellulose (15 H).

TABLE I

(sin $\theta/2$) values from X-ray diffraction patterns for *Dictyostelium* and wood cellulose samples

Sample	Diagram	sin $\theta/2$			
		$(101)_{II}$	$(101)_{I}$	$(10\bar{1})_{I}$	$(002)_{I}$
Dic 1	1	0.1074	0.1310	0.1450	0.190
Dic 2	2	0.1082	0.1310	0.1452	0.191
Dic H	3	0.1059	0.1277	0.1412	0.192
Average lattice plane dist. d (in Å)		7.17	5.92	5.36	4.02
B 2	4	—	0.1288	0.1420	0.194
15	5	0.1057	0.1282	0.1446	0.194
15 H	6	0.1068	0.1284	0.1430	0.194
Average lattice plane dist. d (in Å)		7.24	5.98	5.37	3.96

The d-values were calculated from the Bragg equation $2d \sin \theta/2 = n\lambda$, with $\lambda = 1.5387$ Å and $n = 1$.

mercerized cellulose of a very low crystalline order, even lower than that of wood cellulose which until now has shown the lowest lattice order of all native celluloses investigated (cf. [22–26]). These conclusions about the lattice structure do not agree with Raper's data [20], and the problem will be further studied.

Degree of polymerization.—The degree of polymerization of one of the *Dictyostelium* cellulose samples (Dic 2) and the hydrolyzed sample (Dic H) was determined after nitration using osmotic and viscometric measurements.

To decrease loss of material (the X-ray test samples were used again) all operations in preparing the nitrates were performed in centrifuge tubes.

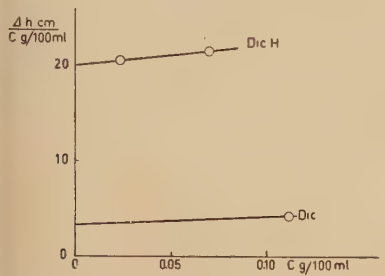
For each sample 5 ml of nitration acid mixture (HNO_3, H_3PO_4 and P_2O_5 in the proportion 48:50:2) was used. The acid was pre-cooled to −16°C and the samples were suspended in the acid under vigorous stirring. The tubes were closed with rubber stoppers and immersed in an ice-bath for 3 hours. The nitration process was stopped by adding 20 ml ice and water under stirring and the nitrate formed immediately recovered and washed repeatedly with distilled water. The nitrates were stabilized by heating in water suspension in an oven at 105°C for two one-hour periods, washed with water, dried in the air and finally in a desiccator over solid sodium hydroxide.

No nitrogen analyses were made because of the small amounts of sample available. The nitrogen content is assumed to be about 13.6 per cent, because wood cellulose samples nitrated in the same way give that value.

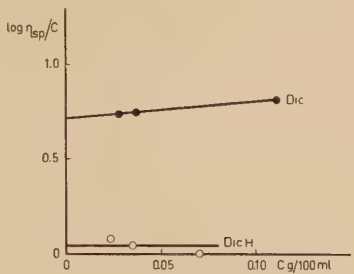
The cellulose nitrate samples were weighed and dissolved in acetone. They were

letely soluble to clear solutions, which were used as such (concentration c_0) after dilution (c_1, c_2, etc.) for viscometric and osmotic measurements.

e osmotic measurements were made using capillary osmometers (Zimm and son [32]) with very tight membranes (Ultracellafilter "Allerfeinst" No. 100 from bran AG, Göttingen, Germany). The osmotic pressures were measured as height rences (Δh in cm) between the menisci of the "inner" and the "outer" (or



Text-fig. 2. Osmotic measurements $\Delta h/c$ vs. c.



Text-fig. 3. Viscometric measurements log η_{sp}/c vs. c.

ence) capillaries (to correct for the capillary rise due to surface tension). The ngs were made using a cathetometer with an accuracy of 0.002 cm. All Δh s were corrected with a constant value −0.020 cm, which was the osmometer ng when the cell was filled with pure solvent. All measurements were made in ame osmometer. The results (Table II) were plotted in diagrams as $\Delta h/c$ vs. c g/100 ml) and extrapolated with straight lines to infinite dilution (Text-fig. 2) g $(\Delta h/c)_0$ values (the slope of the Dic-line was derived from earlier measures for cellulose nitrates in acetone). The average molecular weights (M_n) were lated using the relation

$$\overline{M}_n = \frac{RT}{d(\Delta h/c)_0},$$

e R represents the gas constant, T the absolute temperature (in this case 298.1°K, sponding to 25.0°C) and d the density of the solvent ($d = 0.792$ for acetone). Δh in cm and c in g/100 ml, R has the numerical value 848. A nitrogen content .6 per cent gives a molecular weight of 288 for the nitrated anhydroglucose This means that the average degrees of polymerization ($\overline{DP}_n$) are obtained by ing the M_n values by 288.

TABLE II

Osmotic measurements.

ample	c g/100 ml	Δh cm	$\Delta h_{solvent}$ cm	Δh_{corr}	$\Delta h_{corr}/c$
Dic 2	0.1113	0.492	0.020	0.472	4.24
Dic H	0.0700	1.525	0.020	1.505	21.50
Dic H	0.0232	0.495	0.020	0.475	20.50

The results of the osmotic measurements have been collected in Table II.

TABLE III

$\overline{M}_n$ and $\overline{DP}_n$ values for the original (Dic 2) and hydrolyzed (Dic H) *Dictyostelium* cellulose, both nitrated.

Sample	$\left(\frac{\Delta h\, cm}{c_{g/100\ ml}}\right)_0$	$\overline{M}_n$	$\overline{DP}_n$
Dic 2	3.22	99,130	344
Dic H	20.00	15,960	55

The viscosity measurements were made with a capillary viscometer of the Ubbelohde type with an outflow time (t_0) of 64.5 sec. for pure acetone. The dilutions of the solutions were made with pipette direct in the viscometer and the outflow times (t) were measured after a thorough mixing. The relative viscosity (η_{rel}) for a solution is defined as $\eta_{rel} = t/t_0$ and the specific viscosity as $\eta_{sp} = \eta_{rel} - 1$. The quantities log ($\eta_{sp}/c$) were plotted as a function of c (concentration in g/100 ml) and extrapolated to infinite dilution giving intrinsic viscosity $[\eta]$ (Text-fig. 3). With this procedure $[\eta] = 5.2$ for Dic 2 is obtained. The measurements give no accurate $[\eta]$ value for Dic H. With a horizontal extrapolation line log $[\eta] = 0.04$ and $[\eta] = 1.1 \pm 0.1$. The $\overline{DP}_v$ values are calculated from the intrinsic viscosities using the relation

$$[\eta] = K_m \cdot \overline{DP}_v,$$

where the K_m constant for this system is approximately 10×10^{-3} [7]. The molecular weights ($\overline{M}_v$) are obtained by multiplying the $\overline{DP}_v$ values with 288, which is the molecular weight of the nitrated anhydroglucose unit (Table IV).

TABLE IV

$\overline{DP}_v$ and $\overline{M}_v$ values from viscosity measurements.

Sample	$[\eta]$	$\overline{DP}_v$	$\overline{M}_v$
Dic 2	5.2	520	150,000
Dic H	1.1	110	31,700

The osmotic molecular weights are number averages $\overline{M}_n$ defined as

$$\overline{M}_n = \frac{\sum n_i M_i}{\sum n_i},$$

the viscometric molecular weights in this case weight averages $\bar{M}_w$ ned as

$$\bar{M}_w = \frac{\sum n_i M_i^2}{\sum n_i M_i},$$

re n_i is the number of molecules with the molecular weight M_i. The mation is made over all molecular weights present in the sample.

or a polymolecular or polydisperse substance (containing molecules of rent sizes) $\bar{M}_w$ is always $> \bar{M}_n$. For a statistical distribution of molecular ghts, e.g. a substance containing chain molecules depolymerized at dom, $\bar{M}_w/\bar{M}_n = 2$ is predicted from calculations and verified experimen-. The *Dictyostelium* cellulose shows an $\bar{M}_w/\bar{M}_n$ ratio of 1.5 before and .0 after hydrolysis. The heterogeneous hydrolysis is therefore probably ndom depolymerization of the cellulose chains.

he DP values obtained for the hydrolyzed *Dictyostelium* cellulose are er than those for chemical grade wood celluloses hydrolyzed in the same ($\overline{DP}_n \sim 90$ and $\overline{DP}_v \sim 130$, cf. [21]). This is another indication that the *yostelium* cellulose is more easily accessible (of lower crystalline order) n native wood cellulose.

ine structure.—The isolated framework substance from *Dictyostelium* sisting of the sorophore sheath and the cell walls from sorophore and al disk was investigated with electron microscopy. The specimens were pared on screens covered with Formvar membranes (polyvinylformal) n suspension in distilled water and shadowed with gold-manganese after ersion by ultrasonic irradiation (22 kc., 1–2 watt per sq.cm).

s is also seen in Figs. 13–14 the main part of the sheath is composed of rs with micelle strings oriented parallelly to the axis of the sorophore s. 17 and 18). Besides, the micrographs show the occurrence of parts n a more random orientation of the fibrils. Although it was impossible tudy the fine structure *in situ* (the resolution in the ultrathin sections was sufficient), it may be concluded, from fragments with quite different ntation, that they belong to different layers in the sheath. The fine structure he looser parts of the sheath resembles that of the primary walls of the k and basal disk cells (Fig. 19).

ispersion of partially hydrolyzed substance (Dic H) decomposes the ils to micelles of most varying length, while the width of the micelles is same (about 100 Å) as that of the original strings (Figs. 20 and 21).

SUMMARY

The morphology of cell aggregates of the slime mold *Dictyostelium discoideum* at different stages of development have been investigated as ultrathin sections and studied by electron microscopy (Figs. 1–16).

The investigations were concentrated on the mature sorocarp and especially on the sorophore and its sheath. The resistant framework in the sheath and the cell walls, from earlier work reported to contain cellulose, has been purified and extensively studied using a variety of methods—paper chromatography after hydrolysis, X-ray diffraction (Text-fig. 1), osmotic and viscometric measurements (Text-figs. 2 and 3), and fine structure analysis with electron microscopy after dispersion with ultrasonic waves (Figs. 17–21).

From these investigations the following conclusions have been drawn concerning the sorophore sheath and the cell wall substance:

The resistant fibrous substance, which is deposited extracellularly in the sheath and also occurs in the primary wall of sorophore and basal disk cells, is most probably—as isolated and purified here with hot aqueous 1 per cent sodium hydroxide—a *partly mercerized cellulose* of a very low crystalline order. The elementary fibrils or micelle strings of this cellulose are deposited in the sheath as layers with different orientation. When the substance is partially hydrolyzed, the fibrils are decomposed into rodlike fragments, micelles, of the same width as the original strings ($\sim$ 100 Å) but of length varying within wide limits.

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CHARACTERISTICS OF EXTRACTS FROM LIVER MICROSOMES

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It has frequently been observed that the submicroscopic structures which can be isolated from liver cytoplasm in the form of microsomes are the sites of a particularly rapid incorporation of amino acids into proteins [8, 18, 20]. The microsome material, however, is not homogeneous. By fairly simple extraction procedures it can be separated into soluble subfractions with different rates of amino acid incorporation [9, 10].

In experiments with carcinogenic azo dyes it has been shown that in rat liver these compounds become bound at a particularly rapid rate to the proteins of the microsome material [11, 13]. By use of the same extraction method it was possible even in this case to separate subfractions with different incorporation properties [12].

Preparation of Microsome Extracts

As an introduction to the subfractionation experiments just mentioned [9, 10, 12] a number of methods were tried for the extraction of protein constituents from cytoplasmic particles. Two of these methods were finally preferred, since they combined a satisfactory efficiency with a rather high degree of selectivity.

Extraction of liver microsomes with bicarbonate buffer.—Male albino rats weighing 175–200 g were used. Before the experiments they were kept without food for 12–15 hours. The animals were killed by decapitation, and the livers were rapidly perfused *in situ* with ice-cold 0.25 *M* sucrose containing 0.005 *M* versene. During the perfusion the livers were chilled with ice. The perfused livers were minced and homogenized in the sucrose–versene medium, and microsome fractions were prepared by differential centrifugation essentially as has been described previously [10, 11]. The microsome pellets were washed by centrifugation in 0.25 *M* sucrose.

The microsomes were divided into equal samples, each sample corresponding to approximately 1 g of liver. The samples were suspended in

ml of ice-cold bicarbonate buffer, and were mechanically shaken for
rent periods at low temperature. Afterwards, the samples were diluted
distilled water to 11 ml and centrifuged for 50 minutes at 105,000 × *g*.
contents of ribonucleic acid (RNA) and of protein were determined
he supernatant fluids and in the sedimented residues. RNA was deter-
ed by the method of Ogur and Rosen [16], and the proteins according to

1. Extraction of rat liver microsomes with bicarbonate buffer. Effects of pH, ionic concentra-
and extraction time on the solubilization of ribonucleic acid and protein. Standard con-
ns: 0.1 *N* bicarbonate pH 8.2 for 6 hours.
○———○, RNA. ×———×, protein.

ry *et al.* [15]. The effects of pH, ionic concentration, and extraction time
he amounts of RNA and protein released into the extracts are illustrated
ig. 1.

he investigation of the incorporation of labeled amino acids and carcino-
c azo dyes into cytoplasmic subfractions, which was mentioned in the
oduction [9, 10, 12] was planned on the basis of extraction experiments
his kind. As a standard procedure in the investigation the cytoplasmic
icles were shaken at 1–3°C with 0.1–0.2 *M* bicarbonate buffer pH 8.2–8.4
12–15 hours. After centrifugation the sediments were treated a second
e in the same way, this time for 6 hours. The residues were then further
acted with deoxycholate.

xtraction of microsome residues with deoxycholate.—As is illustrated by
1 the amounts of protein, which are released from microsomes by extrac-
with bicarbonate buffer gradually decrease. When the extracted micro-
e residues are transferred to a solution of deoxycholate (0.4–0.6 per cent,
8.2–8.4) the main part of the material is fairly rapidly dispersed and
ains in the solution even after centrifugation for 45–60 minutes at 105,000
A similar transformation can be observed under the influence of other
rgents, even the nonionic Tween 20.

The Bicarbonate Extracts

The extraction of microsomes with bicarbonate buffer involves an appreciable concentration of solubilized RNA into the extract. As a result of 14 experiments, in which washed microsomes were treated for 15 hours at 0–3°C with 0.2 *M* bicarbonate buffer pH 8.2, on the average 70 (±8) per cent of the RNA were dissolved, while only 25 (±3) per cent of the proteins and 4 per cent of the cholesterol were brought into solution.

The amino acid incorporation experiments [9, 10] as well as the analytical data suggest that the RNA and protein material of the bicarbonate extracts is correlated with the ribonucleoprotein granules of the submicroscopic cellular structures, which have recently been studied by Palade [17] and Littlefield *et al.* [14].

The major part of the extracted nucleotide and protein material could be precipitated by cold 0.2 *N* perchloric acid (Table I). Cold 10 per cent trichloroacetic acid was a slightly less effective precipitant. When bicarbonate extracts were dialyzed in the cold against distilled water (Visking dialysis tubing) considerable amounts of nucleotide material (estimated from their light absorption curves) and of material, probably peptides, reacting with the Folin reagent [15] passed through the membrane.

It is generally believed that RNA-units associated with microsome structures may function like templates in the synthesis of proteins. If this assumption is correct, intermediates in the protein synthesis should be expected to occur in living cells, associated with the RNA of the microsome material. It seemed possible that the

TABLE I

Rat liver microsomes extracted for 15 hours with 0.2 *M* $NaHCO_3$ buffer, pH 8.2. A comparison on a percentual basis of acid-soluble and dialyzable material in the extract. (The Folin-positive material was analyzed by the protein determination method of Lowry *et al.* [15]).

	Per cent acid soluble or dialyzable			
	Total-*N*	Total-P	$\Delta E_{260-300}$	Folin-positive material
Soluble in cold 0.2 *N* perchloric acid	8	10	8	—
Soluble in cold 10 per cent trichloroacetic acid	11	25	—	—
Dialyzable	21	30	27	20

nterial in the bicarbonate extracts, which could be precipitated with perchloric a but passed through the dialysis membrane, might include protein intermediates o this kind. However, in experiments carried out on rats and rabbits *in vivo* the rporation of carboxyl-labeled amino acids was not significantly higher in the ptides of this dialysable material than in the dialyzed proteins of the bicarbonate acts. (After an initial exhaustive treatment of the material with ninhydrin in r to remove free carboxyl groups, the carboxyl carbon involved in peptide bonds w liberated by acid hydrolysis and isolated in the form of $BaCO_3$ by a second ninhydrin treatment.)

The Deoxycholate Extract and Its Change with the State of Nutrition

When deoxycholate extracts of rat liver microsomes were treated under efully controlled temperature conditions with ethanol at different concentions, characteristic precipitation curves could be obtained. The experints illustrated in Fig. 2 were carried out on deoxycholate extracts containing about 7.5 mg protein [15] per ml. Two series of samples were prepled. In one series the extract was used without dilution. The samples of the er series were diluted with an equal volume of water before the ethanol cipitations. The precipitation curves represented in Fig. 2 have reference he series of undiluted samples in each experiment.

he precipitation experiments were carried out according to the method of Askonas The samples of both series were precipitated at the following four standard centrations of ethanol: 26, 31, 35 and 38 per cent. Immediately after the admixe of ethanol the samples were centrifuged for 90 minutes at 21,000 × *g*. The ples with the lower ethanol concentrations were centrifuged at −8°C, the samples the higher concentrations at −12°C. The contents of protein [15], total nitrogen, A [16], and cholesterol [1] were determined in the initial bicarbonate and deoxyate extracts, and in the sediments and supernates obtained from the diluted and iluted samples of the deoxycholate extracts.

s is illustrated by Fig. 2 *B*, the precipitation curves indicated the presence wo components with different ethanol precipitability. One smaller comnent precipitated already at ethanol concentrations lower than 26 per cent. e other component precipitated between 31 and 38 per cent ethanol. The cipitation curves obtained from the analyses of protein, total nitrogen, A and cholesterol were nearly parallel. The total range of deviation been the analyses in the individual experiments is indicated in Fig. 2 by two limiting lines. The somewhat lower precipitability values obtained cholesterol, and in some experiments for RNA, at the higher ethanol centrations may indicate the presence of small amounts of free cholesterol l low molecular nucleotides in these samples.

The precipitation curves obtained from the series of diluted samples were fairly similar to those from the undiluted extracts, illustrated in Fig. 2. As a rule, however, the precipitation values of the diluted series were slightly lower in 26 per cent ethanol and slightly higher in 35 per cent ethanol, when compared with the corresponding values of the non-diluted series.

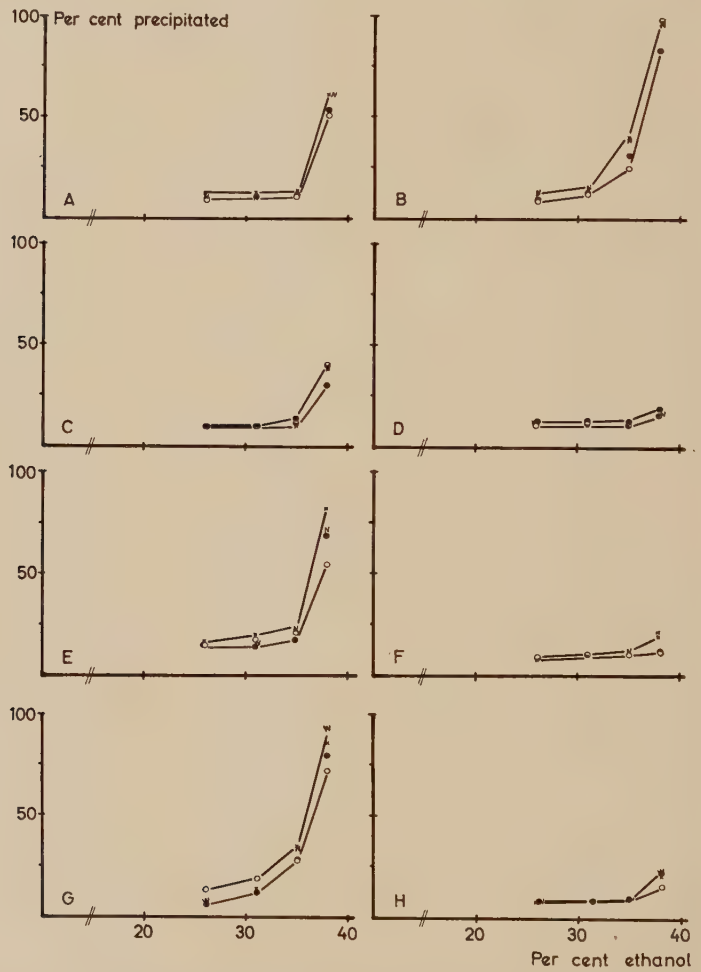

Fig. 2. Precipitation with ethanol of deoxycholate extracts from bicarbonate-treated rat liver microsomes. Effects of nutritive conditions on the precipitability of the extracts.

N, nitrogen; ×, protein; ○, RNA; ●, cholesterol. *A*, without food, 1 hour. *B*, without food, 15 hours. *C*, without food, 48 hours. *D*, without food, 50 hours. *E*, without food, 6 days. *F*, like *B*, but 3 ml of olive oil received 24 hours before decapitation. *G*, like *B*, but rat maintained on *m'*-DAB diet for 5 weeks. *H*, like *F*, but 60 mg *m'*-DAB dissolved in the olive oil.

When rats which had been kept without food for different periods were used in experiments of this kind, pronounced differences were observed in the precipitability of the second component of the deoxycholate extracts. The precipitability was highest in those rats, which had been kept without food for 15–20 hours (Fig. 2*B*). Animals which had been maintained under normal feeding conditions until shortly before the start of the experiment (Fig. 2*A*) showed a lower precipitability of the deoxycholate extracts. The decreased ethanol precipitability was still more striking in the case of animals which had been kept for 2–3 days without food. After prolonged starvation a higher precipitability of the deoxycholate extracts was again observed (Fig. 2*E*). Since it is known that the lipid content of the liver increases during the early stages of starvation [4], it seemed possible that the decreased precipitability of the deoxycholate extracts might be correlated with a higher content of lipid components in such extracts. The relative amounts of cholesterol which were observed in experiments *C* and *D* were only insignificantly lower, however, than the amounts observed in experiment *B*. On the other hand, a rat which had been kept without food for 15 hours after a previous single dose of olive oil (2 ml introduced into the stomach, and 1 ml into the abdominal cavity, cf. [11]) showed a very low precipitability of the deoxycholate extract (Fig. 2*F*). The administration of a carcinogenic azo dye, 3'-methyl-4-dimethylaminoazobenzene by way of diet (Fig. 2*G*) or as a single dose (60 mg dissolved in 3 ml of olive oil [cf. 11]) did not significantly change the precipitability of the deoxycholate extracts (Fig. 2*H*), in spite of the fact that this dye becomes bound to the proteins of this subfraction at particularly rapid rate [12].

In the experiments reported previously [9, 10, 12] on the incorporation of labeled amino acids and carcinogenic azo dyes in the subfractions of cytoplasmic particles similar ethanol precipitations were performed. The ethanol precipitates were in these experiments allowed to settle for 3–4 hours before centrifugation, and the precipitation steps were carried out successively in the same extract. Under these conditions somewhat lower concentrations of ethanol could be used at the fractionations. A more satisfactory equilibration was obviously obtained in this way, but probably at the expense of a higher degree of denaturation of the protein complexes (cf. next section).

The Distribution of Enzymes in the Microsome Subfractions

Glucose-6-phosphatase.—The activity of glucose-6-phosphatase was determined according to de Duve *et al.* [5]. After the extraction with bicarbonate buffer, over 95 per cent of the activity remained in the insoluble residues.

When these residues were treated with cold 0.4 per cent deoxycholate for 90 minutes, the enzymatic activity of the system decreased to approximately 20 per cent. No ethanol fractionations were therefore undertaken.

DPNH- and TPNH-cytochrome c reductases.—These enzymes were determined by the methods of Hogeboom and Schneider [6, 7]. Even in this case the enzymatic activities remained almost exclusively in the residues (90 and 85 per cent, respectively), when microsomes were extracted with bicarbonate buffer. The activity of the DPNH-cytochrome *c* reductase in the residue was nearly 10 times higher than the activity of the TPNH-cytochrome *c* reductase [cf. 19]. When the extracted microsome residues were treated with deoxycholate, the major part of the activities passed into solution. On ethanol fractionation, however, the activities of both enzymes were seriously diminished.

DPNH- and TPNH-diaphorases.—The enzymatic activities were determined in analogy with the cytochrome *c* reductase activities, 2,6-dichlorophenolindophenol being used as electron acceptor in this system instead of cytochrome *c*. As might be expected, the diaphorases were extracted under the same conditions as the corresponding cytochrome *c* reductases. They were precipitated with ethanol at low temperatures without appreciable loss of activity. Typical precipitation curves for the enzymes are shown in Fig. 3. Under the precipitation conditions described, the enzymatic activities were not significantly concentrated to any particular fraction. The TPNH diaphorase, however, usually showed a slightly higher precipitability at low ethanol concentration than the DPNH-diaphorase.

The experiment illustrated in Fig. 3 was carried out with a rat on standard diet, which had been kept without food for 15 hours (cf. Fig. 2*B*). When a rat, which had not been fasting, was used in a similar experiment, only 10 per cent of the DPNH diaphorase and 12 per cent of the TPNH diaphorase were precipitated from the dioxycholate extract at a final ethanol concentration of 40 per cent.

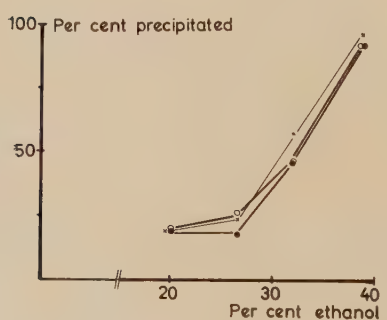

Fig. 3. Precipitation with ethanol of DPNH- and TPNH-diaphorases in deoxycholate extracts from rat liver microsomes, compared with the precipitation of total protein.
●———●, DPNH diaphorase.
○———○, TPNH diaphorase.
×———×, protein.

n the basis of the enzyme experiments and the analytical data mentioned he previous section it is considered that the microsome residues are ersed by deoxycholate in the form of fairly complex units. It is not wn at present to which differences in the chemical or physical nature nese complexes the ethanol precipitation curves are correlated. Electro- retic analyses of deoxycholate extracts, performed in this laboratory by T. Äyräpää have shown that the electric charge of the colloidal units is ely determined by the deoxycholate ions stabilizing the system. Electro- retic separation of the microsome material dissolved in deoxycholate tion is therefore not practicable. The use of nonionic detergents may ibly prove more satisfactory in this respect.

SUMMARY

hen rat liver microsomes are treated at 1–3°C with 0.1–0.2 *M* bicarbonate er, pH 8.2–8.4, about 70 per cent of the ribonucleic acid is gradually ased from the particles together with some protein.

he extracted microsome residues can be largely brought into solution by ment with deoxycholate or other detergents. The properties of this ersed material varies pronouncedly with the nutritive state of the animal. precipitability of the extracts with ethanol increases during the first rs of starvation. After 2–3 days it is very low, but later it increases again. variation is correlated with the fat mobilization during starvation, and probably due to an increased lipid content in the material.

mponents with different ethanol precipitability occur in the deoxycholate acts. Their chemical composition is, however, fairly similar, as regards ein, ribonucleic acid, cholesterol and two enzymes, DPNH- and TPNH- horases. The microsome residues are evidently dispersed by deoxy- ate in the form of fairly complex colloidal units.

he methods considered in this report have been employed in experiments he incorporation of labeled amino acids and carcinogenic azo dyes into ractions of cytoplasmic particles from liver. These experiments have been lished previously.

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PROPAGATION OF L STRAIN MOUSE CELLS IN SUSPENSION[1]

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ANIMAL cells of many types, when introduced into a flask as a suspension in nutrient medium, will adhere to the bottom of the flask and proliferate as a layer on the glass surface. In recent years, this has become a common method of propagating animal cells *in vitro*, and Earle and his associates [1, 2, 22] have introduced short term replicate culture techniques to facilitate quantitative studies with such cells. Nevertheless, this type of system does not lend itself well to many biochemical studies with animal cells. In an effort to provide a better system for such studies, a method for propagating a strain of monkey kidney cells in suspension was developed recently by Graham and Siminovitch [9]. Essentially, the technique was similar to that first used by Earle and his associates [5] for cultivating mouse connective tissue cells (L strain) in suspension. Certain technical difficulties, which the earlier workers encountered, were, however, surmounted in the experiments with the monkey kidney strain. It proved possible to propagate the monkey kidney cells without an initial lag phase, and achieve a 100-fold increase in population during the logarithmic phase of multiplication. The cells showed no tendency to clump or stick to the walls of the tube.

Since the results with monkey kidney cells were reported [9] several other cell types have been cultivated by the same method. In this paper we describe some of the factors that influence the kinetics of multiplication of L cells when propagated in suspension by the rotating culture technique.

MATERIALS AND METHODS

Cells.—The L strain of cells, which was used throughout, was derived originally from adult mouse connective tissue by Earle and his associates, and later continued as the progeny of a single cell [21].

Medium.—A chemically defined mixture of amino acids, vitamins, coenzymes, and other metabolites, in a balanced salt solution containing glucose, was supplemented with horse serum. The defined mixture was developed in the laboratory of

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Fig. 1. Appearance of L cells cultivated in suspension. ×215.

Dr. R. C. Parker [20] and is referred to as solution CMRL-1066; it differs from solution 858 [12] in that it contains thiamine, riboflavin, niacin, niacinamide and pantothenate in the concentrations specified earlier for solution 703 [11], but does not contain cholesterol, Tween 80, or vitamins A, D, K, E. For routine use, the medium consisted of 80 per cent solution CMRL-1066 and 20 per cent horse serum and this mixture will be referred to as "medium A". Other media were used for some experiments and will be described later. All media were passed through a Seitz filter under pressure before use.

Propagation of cells.—L cells were maintained, in medium A, in Kolle flasks where they multiplied while adhering to the glass surface.

To cultivate the cells in suspension, the fluid in a Kolle flask culture was replaced with fresh medium, part of the cell layer was scraped off, and 15 to 20 ml of the resulting suspension was placed in a pyrex test tube (6 inches × 1 inch diameter) closed with a silicone stopper.[1] The tube was shaken vigorously several times and fixed onto a large wooden wheel with its long axis parallel to the axis of rotation of the wheel. The wheel was rotated mechanically at 45 to 50 rpm at 37°C. At intervals thereafter 1 ml samples were withdrawn and the cells were stained by the addition of a drop of a 1 per cent aqueous solution of brilliant cresyl blue and enu-

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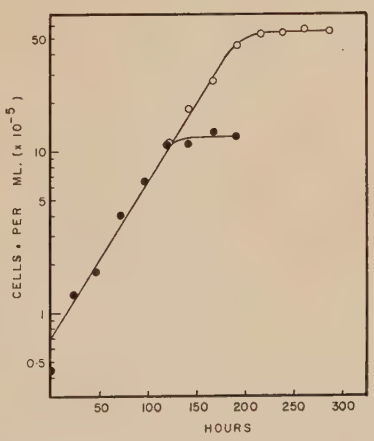
ted in a Petroff–Hausser bacterial counter. Within a few hours after rotation e culture was begun the cells became spherical in shape. When the initial cell entration was between 5×10^4 and 10^6 per ml the cells entered the logarithmic e of multiplication within 24 hours. As described later L cells could be kept nuously in the logarithmic phase in rotating cultures, and such cells were used cula for all the experiments described in this paper. Cells cultivated in suspension spherical in shape as shown in Fig. 1. If a small quantity of such a culture was d in a stationary tube or flask the cells settled, adhered to the glass surface, and iplied to form a continuous layer in the usual way.

EXPERIMENTAL

aximum cell density in rotating culture.—Earlier work with a strain of key kidney cells [9] demonstrated that, in a rotating culture the cells iplied at a logarithmic rate to a density of about 3×10^6 cells per ml, which the population density remained constant for several days. n the medium was renewed at suitable intervals, after the population ity reached 3×10^6 cells per ml, the cells multiplied to a concentration bout 10^7 per ml. Essentially similar results were obtained with L cells nown by the following experiment.

rty milliliters of medium A at 37°C were inoculated to a concentration $\times 10^4$ L cells per ml from a stock culture in the logarithmic phase of th, and rotation of the culture was begun immediately. Cell counts were at intervals. As shown in Fig. 2, cell multiplication began immediately continued until the population reached about 1.2×10^6 cells per ml. At point the culture was divided into two. Rotation of one portion was inued as before. The remainder was centrifuged at about 1000 rpm for



. Increase in population of L cells propagated in sion in a mixture composed of 80 per cent solu- MRL-1066, 20 per cent horse serum. Open circles, m renewed at times indicated by the circles; circles, medium not renewed. Generation time logarithmic phase was 29 hours.

5 minutes, the supernatant withdrawn and replaced with fresh medium at 37°C. Rotation of this culture was begun immediately, and at 24-hourly intervals the medium was renewed in a similar manner. It is seen that if the medium was not replaced with fresh nutrient, the cells multiplied at a logarithmic rate to a density of about 1.3×10^6 cells per ml, after which the population remained constant. A 20-fold increase in cell number was thereby obtained. If the medium was replaced at intervals after the culture reached a concentration of 10^6 cells per ml, multiplication continued to about 5×10^6 cells per ml. It was thus possible to achieve a 70-fold increase in cells (6 generations) in the logarithmic phase, with a generation time of about 30 hours.

The factors that determine the final population density in a rotating culture are not yet known. It is known, however, from the experiment just described that lack of oxygen and overcrowding of cells do not limit multiplication at cell concentrations below 4×10^6 per ml. The pH of cultures, even at the higher cell concentrations, seldom falls below 7 and, therefore, is not the limiting factor. Possibly, toxic products which inhibit cell multiplication accumulate during growth of the culture, or the medium becomes depleted of some essential nutrient.

When L cells were subcultured from rotating tubes into fresh medium in other tubes, at any time during the logarithmic phase, multiplication in the new rotating culture commenced without a lag when the initial concentration was 4×10^4 cells per ml or greater. Cells which had been in stationary phase for not more than a day would also commence to multiply at a logarithmic rate on subculture. Consequently, a stock culture of L cells could be kept continuously in the logarithmic phase by replacing part of the culture at intervals with fresh medium, in order to maintain the cell concentration between 10^5 and 10^6 per ml. Cells taken from such a stock culture at intervals over a period of nine months, and inoculated into fresh medium, multiplied without an early lag with a generation time of 26 to 30 hours.

Effect of size of inoculum on final population density.—It was shown in the previous experiment that cells, subcultured into fresh medium, multiplied to a population density of about 1.2×10^6 cells per ml if the medium was not renewed. While investigating the effect of initial cell concentrations on multiplication in rotating cultures, it was observed that the final population density was a function of the initial density. This point is illustrated by the following experiment:

Four 20 ml aliquots of medium A at 37°C were inoculated with cells from a logarithmic phase stock culture to give final concentrations of 1.6×10^5, 3.1×10^5, 6.8×10^5, and 1.1×10^6 cells per ml respectively, and rotation on

culture was begun immediately. As shown in Fig. 3, higher final cell ntrations were achieved as the initial concentrations were increased. rtheless, it will be observed that, if the initial cell densities are subtracted the final densities, the number of cells produced was approximately ame in each culture, 1.2×10^6 cells per ml. It would appear that this ase in cell number is about the maximum that can be obtained using

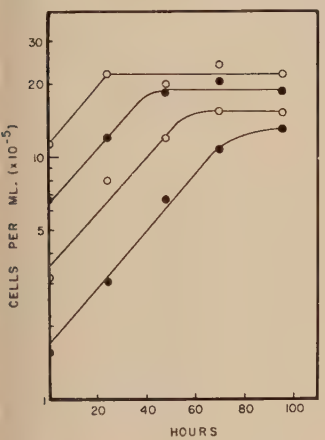


Fig. 3.

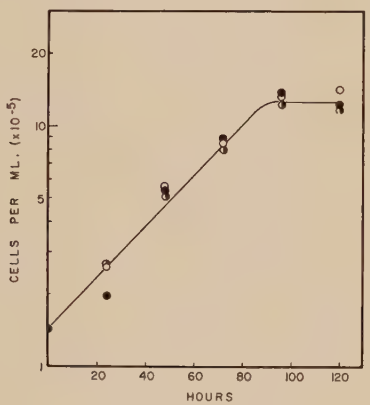


Fig. 4.

Effect of initial cell density on size of final population achieved in rotating cultures of s. Generation times, 27 hours.

Effect of dialysis of horse serum on multiplication of L cells in suspension. For each culture ed horse serum was mixed with solution CMRL-1066 to a concentration of 10 per cent. ○ serum dialysed for 1 day; ●●● serum dialysed for 3 days; ◑◑◑ serum dialysed for 5 Generation time 29 hours.

um A. Earle *et al.* [4] have observed 4×10^4 cells per ml to be about inimum concentration that would consistently initiate cell proliferation ationary cultures. While we have not determined the lowest cell con- ation that could be used to seed rotating cultures, an initial cell density of $> 0^4$ cells per ml regularly resulted in logarithmic growth.

Effect of dialysis of horse serum on cell multiplication.—The following ex- ment was designed to show whether L cells would multiply in suspen- in the presence of dialysed horse serum. A quantity of horse serum was d in a 1 inch diameter cellophane bag and dialysed against 0.9 per cent m chloride solution containing 100 micrograms of streptomycin per ml. saline solution was changed twice daily and the contents of the bag were d frequently. At daily intervals, for five days, a portion of the serum withdrawn and added to solution CMRL-1066 to a concentration of

10 per cent. Twenty ml of each of the five mixtures was inoculated with L cells to give a concentration of 1.5×10^5 cells per ml, and the cultures were rotated. Fig. 4 shows the increase in population for the cultures containing horse serum which had been dialysed 1, 3 and 5 days respectively. Results with the other two cultures were similar to those shown but are not plotted for the sake of clarity. The generation time and final cell density in each

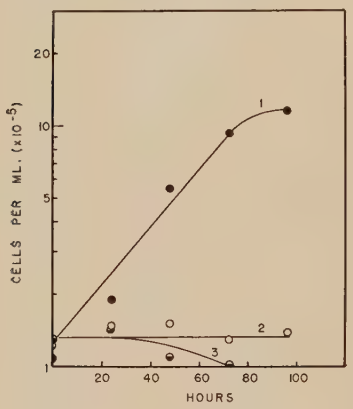


Fig. 5. Effect of omission of glucose or methionine from the medium on multiplication of L cells in suspension. Curve 1 control culture containing 10 per cent dialysed horse serum 90 per cent solution CMRL-1066. Generation time, 25 hours. Curve 2, same conditions as for Curve 1 but methionine omitted from the medium. Curve 3, same conditions as for Curve 1 but glucose omitted from the medium.

culture were similar to those normally obtained using undialysed horse serum. It is evident, therefore, that dialysis of horse serum for 5 days has no effect on its ability to support the multiplication of L cells when the serum is used to 10 per cent concentration in the synthetic mixture. The nutritional properties of horse serum apparently do not reside in dialysable constituents.

Effect of media deficient in methionine or glucose on the multiplication of L cells.—Eagle [3] has recently defined the essential amino acid requirements for L cells propagated on glass surfaces in the presence of dialysed horse serum. He found, for example that if any one of twelve amino acids was left out of the medium no cell proliferation occurred. We have performed the following experiment to find what effect the omission of methionine, found by Eagle to be an essential amino acid, would have on the multiplication of L cells in suspension. The growth of L cells in glucose-free medium was also studied.

Synthetic mixtures were prepared without glucose or methionine and supplemented with horse serum, which had been dialysed for 3 days, to a concentration of 10 per cent. An aliquot of each medium was inoculated with L cells to give a concentration of about 1.3×10^5 per ml and the cultures were rotated. Fig. 5 shows the results with the two cultures and a third, control

ure, which contained the complete synthetic mixture (CMRL-1066) with 1 per cent dialysed horse serum. While the control culture grew in the u l way there was no cell multiplication in either the methionine-free o ne glucose-free medium. In fact, there was a progressive decrease in cell n ber in the glucose-free medium after 24 hours.

ncrease in ribo- and desoxyribonucleic acids and protein during cell multipli- on.—In the previous experiments some of the factors that influence the k tics of multiplication of L cells in suspension have been elucidated. Before it system could be used for projected studies of nucleic acid and protein m abolism, however, it was important to determine whether these cellular co stituents increased at the same rate as the cell population during the lo rithmic phase. Using the replicate culture procedure to propagate L cells, H ly *et al.* [10] have already shown that desoxyribonucleic acid (DNA) p sphorus increased at the same rate as the number of cell nuclei. The fo wing experiment demonstrates that, in a rotating culture of L cells, the a unts of ribonucleic acid (RNA) and DNA per cell remain constant d ng the period of cell multiplication.

ighty milliliters of medim A were inoculated with L cells to a concentra- i of 1.5×10^5 per ml and the culture was rotated at 37°C. At zero time, and at ntervals thereafter, aliquots were removed, in duplicate, for nucleic acid a ysis, and further samples were removed for enumeration of cells. F maldehyde was added to each of the samples for analysis, to a final con- c ration of 1 per cent and the cells were centrifuged. The supernatant was di arded and 5 ml of ice-cold trichloroacetic acid (TCA) was added to the se ment. This mixture was stirred, allowed to stand at 4°C for 15 minutes, a again centrifuged. The residue was washed once with ice-cold absolute et nol and extracted twice with boiling ethanol for 15 minute periods. 2. ml of 5 per cent TCA was added to the sediment, the mixture was heated at 0°C for 20 minutes to extract nucleic acids and again centrifuged. The h TCA extraction procedure was repeated on the sediment, the two extracts w e combined and the volume was adjusted to 50 ml. Two 2 ml aliquots w e removed from each extract; DNA was determined on one by the method o eriotti [1] and RNA on the second by the method of Mejbaum [14] as m ified by Ogur and Rosen [16]. The results showed (Fig. 6) that the a unts of nucleic acid ribose and desoxyribose, measured by the color r tions for these sugars, increased at the same rate as the cell population d ng the logarithmic phase.

or the experiment just described the color equivalent scale (Fig. 6) was st dardized by the Ceriotti reaction for desoxyribose [1] against a sample

of thymus DNA of known phosphorus content. Ten color units are equivalent to 1.3 micrograms of DNAP. Each cell contains, therefore, 1.3×10^{-6} micrograms of DNAP during the logarithmic phase. Direct phosphorus estimation on the DNA fraction of logarithmic phase L cells, after separation by the Schmidt and Thannhauser procedure [23], also resulted in the value of 1.3×10^{-6} micrograms of DNAP per cell, in agreement with result of Healy *et al.*

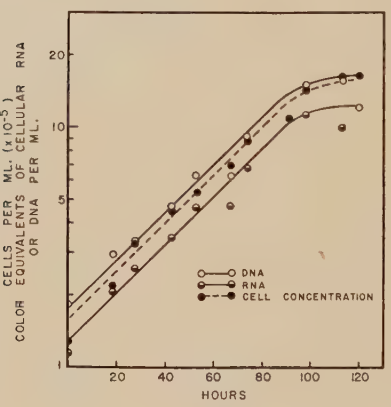


Fig. 6.

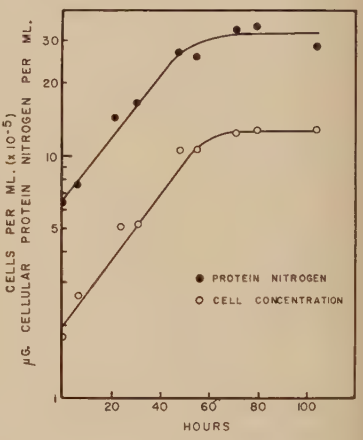


Fig. 7.

Fig. 6. Increase in population, RNA, and DNA, of L cells propagated in suspension. The standardization of the color equivalent scale is described in the text.
Fig. 7. Increase in population and cellular protein nitrogen of L cells propagated in suspension. The standardization of the protein nitrogen scale is described in the text.

[10] for L cells cultivated in stationary flasks. The color equivalent scale of Fig. 6 was also standardized by the orcinol reaction for ribose [16] using adenosine as the reference standard. On the assumptions that purine ribosides comprise half the total ribosides in cellular RNA, that only purine ribosides react in the orcinol test, and that purine linked ribose in cellular RNA develops the same color density, mol for mol, as the standard adenosine solution, it was calculated that each L cell contained 2.9×10^{-6} micrograms of RNAP.

Oyama and Eagle [17] have recently measured the protein content of several cell strains propagated by a replicate culture procedure. We have applied their modification of the method of Lowry *et al.* [13] to determine the rate of increase in cellular protein during the multiplication of L cells in suspension. Fifty milliliters of medium A were inoculated with L cells to a density of 1.5×10^5 per ml, and the culture was rotated at 37°C. Samples

e withdrawn at intervals for cell enumeration and protein analysis. The s-ples for analysis were centrifuged and the cells were washed with syn- t ic medium containing 2 per cent formaldehyde and then with Hank's s ution. Protein estimations were carried out on the washed cells and the r ults are shown in Fig. 7. It will be observed that the amount of protein p cell remained constant during the logarithmic phase of multiplication. The standard of reference for the protein estimations shown in Fig. 7 w a solution of human serum albumin, essentially the fraction V described b Cohn *et al.* [2], for which the nitrogen content was determined by the K ldahl method. From Fig. 7 it is evident that L cells in the logarithmic p se contain an amount of protein nitrogen per cell equivalent to 3.28×10^{-5} n rograms of human serum albumin nitrogen. L cells cultivated by a r icate culture procedure in stationary flasks were found to contain the e ivalent of 4.28×10^{-5} micrograms of serum albumin nitrogen per cell.

DISCUSSION

n addition to L cells, and the monkey kidney strain described earlier [9], t other cell strains have been propagated in suspension with the rotating c ure technique. All of these strains arose as a result of marked morphologic and physiological alterations [18, 19] in the original cell lines following p longed maintenance by Dr. R. C. Parker and his associates. Three cell li s were derived from monkey kidney, three from human bone marrow, o from mouse liver, one from Hela cells [8], one from the 14 *p* strain of n mal rat connective tissue [7], and one from a human foreskin strain (FS4) is ated by Swim and Parker [24].

s we have shown in this paper for L cells, the kinetics of multiplication of th various cell strains, cultivated in suspension, resemble those of bacteria l. Such animal cell systems may be used conveniently for many biochemic studies. Quantitative assay of cell numbers is simple and accurate; u form sampling is assured since there is no tendency for the cells to clump a the cell suspensions are homogeneous; a variety of determinations can b carried out on a single culture; the cells may be kept in the logarithmic p se for an unlimited number of generations and biochemical events can, th efore, be observed over long periods in cells maintained in a physiologic y defined state. Further, we have observed that vaccinia and herpes simplex vi ses will multiply in, and lyse, L cells propagated in suspension. A virus– h system is thereby provided, for studies of animal virus multiplication, th is analogous to the virulent bacteriophage–bacterium system.

SUMMARY

A technique is described for cultivating Earle's L strain mouse cells in suspension in a chemically defined solution supplemented with horse serum. The increase in population density was about 1.3×10^6 cells per ml during the logarithmic phase if the medium was not renewed. The generation time was about 28 hours. Renewal of the medium at suitable intervals permitted the population to increase exponentially to a density of 5×10^6 cells per ml. L cells could be kept indefinitely in the logarithmic phase by frequent subculture. Dialysed horse serum added to the defined solution supported cell multiplication as well as an equal amount of undialysed serum. If either methionine or glucose was omitted from the medium containing dialysed serum no cell proliferation occurred. During the logarithmic phase of multiplication the amounts of RNA, DNA and protein per cell remained constant.

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THE CYTOCHEMICAL MORPHOLOGY OF THE PRODUCTION OF ANTIBODY[1]

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present paper is the last in a series [27, 28, 33, 34, 50, 51] which emphasizes the experimental, quantitative, and terminological aspects of the cytological response to antigen. We wish to review briefly the information which led to this series since the recent reviews of the subject are organized quite differently.

Three separable lines of investigation gave rise to the essentials of our present knowledge of the sites of antibody formation: (1) examination of the blood-forming organs during infection and immunization, (2) definition of plasma cells in sites of inflammation and during immunization, and (3) association of plasma cells with high concentrations of circulating globulins.

Examination of the blood-forming organs during infection and immunization revealed high concentrations of antibody in the spleen during the early days of immunization, enlargement of the spleen and lymph nodes during infection, and phagocytosis of particulate antigens by reticulo-endothelial cells [14, 30]. These observations led, respectively, to the demonstration that the rabbit spleen has a role in the production of circulating antibody [32], a definition of the principal cell type involved [40], and direct evidence that the plasma cells contain large amounts of antibody [9].

In 1890 the "cianophil cells" of the external condylomata of syphilis were described as having an ellipsoidal or spherical shape, an eccentric and spherical nucleus with a distinct chromatic network, and a cytoplasm which stained with aniline dyes and contained vacuoles [39]. In 1891 the same cells were described in the dermal tubercles of case of tuberculosis and were named "Plasmazellen" because of their abundant intensely stained cytoplasm [45]. (The intent would be more obvious if the "Plasmazellen" were referred to in English by the correct translation which is "cytoplasm cells".) In 1895 the presence of plasma cells in the apparently normal tissues of several animals and their relative abundance in infected tissues were emphasized [46]. Proliferations of plasma cells were intentionally induced in rabbits by subcutaneous injections of $\frac{1}{4}$ to $\frac{1}{2}$ g of tuberculin and large numbers of the cells were found in the spleen on the second and fourth days after injection [46]. As a result of exploratory observations reported in 1930 [43], the first photographic proof of the induction of

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large proliferations of plasma cells by "tuberculo-proteins" was published in 1931 [31]. This report of the proliferation and differentiation of plasma cells in response to a non-infectious agent has been confirmed [17, 28].

Marschalko's results [46] are buried in a long paper which emphasizes the similarity of the nuclei of pycnotic plasma cells and pycnotic lymphocytes and uses this similarity as evidence of the derivation of the former from the latter. This point of view, and the terminology associated with it, are common in the literature, but only one reference to his experimental work with tuberculin was found, and this was brief and non-critical. It remained for Miller, in apparent ignorance of Marschalko's experiments with tuberculin, to demonstrate very simply that some pycnotic plasma cells are derived from large blast cells [31]. While Marschalko's contention that some pycnotic plasma cells are derived from pycnotic lymphocytes may still have a basis in the reports of two size classes of plasma cells [17], Miller demonstrated that Marschalko's contention could not be valid for all pycnotic plasma cells. Miller's work is referred to occasionally [42, 47], but no adequate appreciation of its main significance has been found. This and corollary reports by Wiseman [48, 49], also stimulated by Florence Sabin, are sometimes dismissed so abruptly that the reader is not likely to realize that they are experimental papers.

It seems likely that the basic reason for the lack of appreciation of the immunological aspect of Marschalko's and Miller's experiments was due to their use of tuberculin, instead of some other protein mixture, since there was until recently no apparent relation between tuberculin sensitivity and the concentration of detectable serum antibody [20]. Apparently Rich was the first worker to bypass this difficulty by use of antigenic proteins from noninfectious sources [40].

In 1900 it was astutely recognized and demonstrated, at a time when only a few cases had been reported, that the plasma cells were the definitive cells of the tumors of multiple myeloma, as defined by roentgenograms [52]. In 1928 hyperglobulinemia in a case of plasmacellular multiple myeloma was reported [38] and by 1937 ample clinical evidence suggested that the plasma cells were a source of serum globulins [4]. Since antibodies were known to be globulins, attempts were made to induce proliferations of plasma cells by injections of antigens. Photographic proof of the proliferation of plasma cells induced by injections of pneumococci was published in 1941 [6]. Subsequently the frequencies of immature plasma cells were correlated with the production of antibody in immunized adult rabbits, in cultures of spleen tissues from immunized adult rabbits [15, 16, 17], and in young rabbits which received spleen tissues from immunized adult rabbits [18].

The definitive characteristic of all stages of plasma cell proliferation is the cytoplasmic basophilia which, contrary to that of most other cells, becomes more intense with age. As early as 1913 this basophilia was associated with nucleoprotein [36]. By 1938 the morphology and widespread occurrence of basophilia in cells of exocrine glands had been critically and synthetically examined [41] and in 1941 [8] a positive relation was established between high concentrations of protein and pentose nucleic acid in the cytoplasm of many cells. In 1945 it was reported that the cytoplasm of plasma cells absorbed intensely in the ultra-violet, except for an area next to the nucleus, and this absorption was greatly diminished by ribonuclease [5]. This constitutes as yet the best evidence that the plasma cells are intensively engaged in protein synthesis.

though plasma cells have been reported from fish, amphibia, reptiles, birds and mals [11], most of the significant studies have been performed on mammals. ever, the chicken is the only animal in which quantitative changes in cell popula-, nucleic acids and circulating antibody have been examined statistically follow-ntigen injection [28]. The basis for these quantitative determinations was laid in a series of statistical studies in which (1) the increase with age in the ability to ace antibody was estimated [50], (2) the increase in spleen weight during normal th was determined and associated with the increase in ability to produce antibody (3) the effect of splenectomy on antibody production was found to be dependent the amount of antigen injected [51], (4) the magnitude and time course of the ase in spleen weight induced by intravenous injection of antigen was described and (5) the moderate antibody and cellular responses of the six weeks old chicken, relatively small amount of antigen, were examined [27]. One of the more im-nt aspects of this series is the utilization of the definitions and nomenclature of Committee for Clarification of the Nomenclature of Cells and Diseases of the d and Blood-Forming Organs" which classifies plasma cells as plasmablasts, lasmacytes, and plasmacytes according to their stage of development [35]. In non with others we have never been able to distinguish plasmablasts from any blast cell and refer to all such cells simply as blast cells. Finally it should be asized that the committee's definitions are based upon dried human blood rs and our work has been mostly with wet-fixed chicken imprints and sections h show fewer obvious distortions and classical tinctorial distinctions than the smears.

The object of the present observations is to relate cytochemical changes the intravenous injection of antigen. Since the outstanding chemical ges are the increases in spleen pentose nucleic acid and circulating body protein [28] and the outstanding cellular change is the increase in ma cells [28] which contain large amounts of pentose nucleic acid [5] and ohydrate [37, 47], cytochemical methods for pentose nucleic acid, pro- and carbohydrate were applied. Because the different stages of plasma development are relatively easy to recognize there is no difficulty in lating the cytochemical changes with the stage of development qualita-y. Direct correlation with antibody protein synthesis is not attempted in study, but a correlation can be deduced by comparison with the results the previous statistical studies [28].

METHODS AND MATERIALS

ssue samples were obtained from the spleens of 30 six to nine months old chickens th sexes from two to ten days after a second intravenous injection of 250 mg serum albumin per kg body weight. The cells were readily identified on the basis evious studies [27, 28]. Imprints and two micra sections, from frozen-dried tis- were fixed in calcium formol [1]. Imprints were never dried. Carbohydrate was

recognized by the periodic acid–Schiff method with non-oxidized, acetone-extracted and diastase controls [21], nucleic acid by Azure B [19], and protein by Brom phenol blue [29]. The methods of preparation used were selected for minimal cytological distortion and are not as conducive to subsequent enzyme treatment as some of the more disruptive methods. A number of observations made with other techniques are not emphasized because of the difficulties of interpretation.

RESULTS

The results of the three cytochemical methods are each presented in the following manner: the previous use of the technique with plasma cells, the chemical significance of the technique, our observations, and the relation of our observations to plasma cell development.

The cytoplasm of plasma cells has been reported to color erratically with the periodic acid–Schiff method, and the Russell Bodies, which often accompany plasma cells, have been reported to color consistently and intensely [37]. A positive reaction is indicative of glycol and/or mono-amino glycol groups and is characteristic of many carbohydrates [21]. However, a negative reaction is not proof that glycol groups or carbohydrates are absent since not all the glycol groups of some carbohydrates react [23]. The controls employed here are sometimes said to make the method specific for polysaccharides [21], but this seems questionable [25]. In this study, the cytoplasm of plasma cells colored strongly and the Russell Bodies colored intensely. Figs. 1, 2, and 3 illustrate the reactions of blast cells, proplasmacytes, and

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All photographs were taken with 4 × 5 inch Kodak Metallographic (orthochromatic) or M (panchromatic) plates, developed in Kodak D-19, and printed and developed for highest contrast. A standard ribbon filament lamp and Wratten filters were used with a 90 × apochromatic objective of 1.30 numerical aperture, and a 12.5 × compensating ocular, and with a Spencer 97 × achromatic dark M phase contrast objective of numerical aperture 1.25, and a 10 × ocular. The magnifications of the prints are 4500 to 5000 × before reduction for publication. Except where otherwise noted, all prints were obtained with a No. 74 green filter and apochromatic optics.

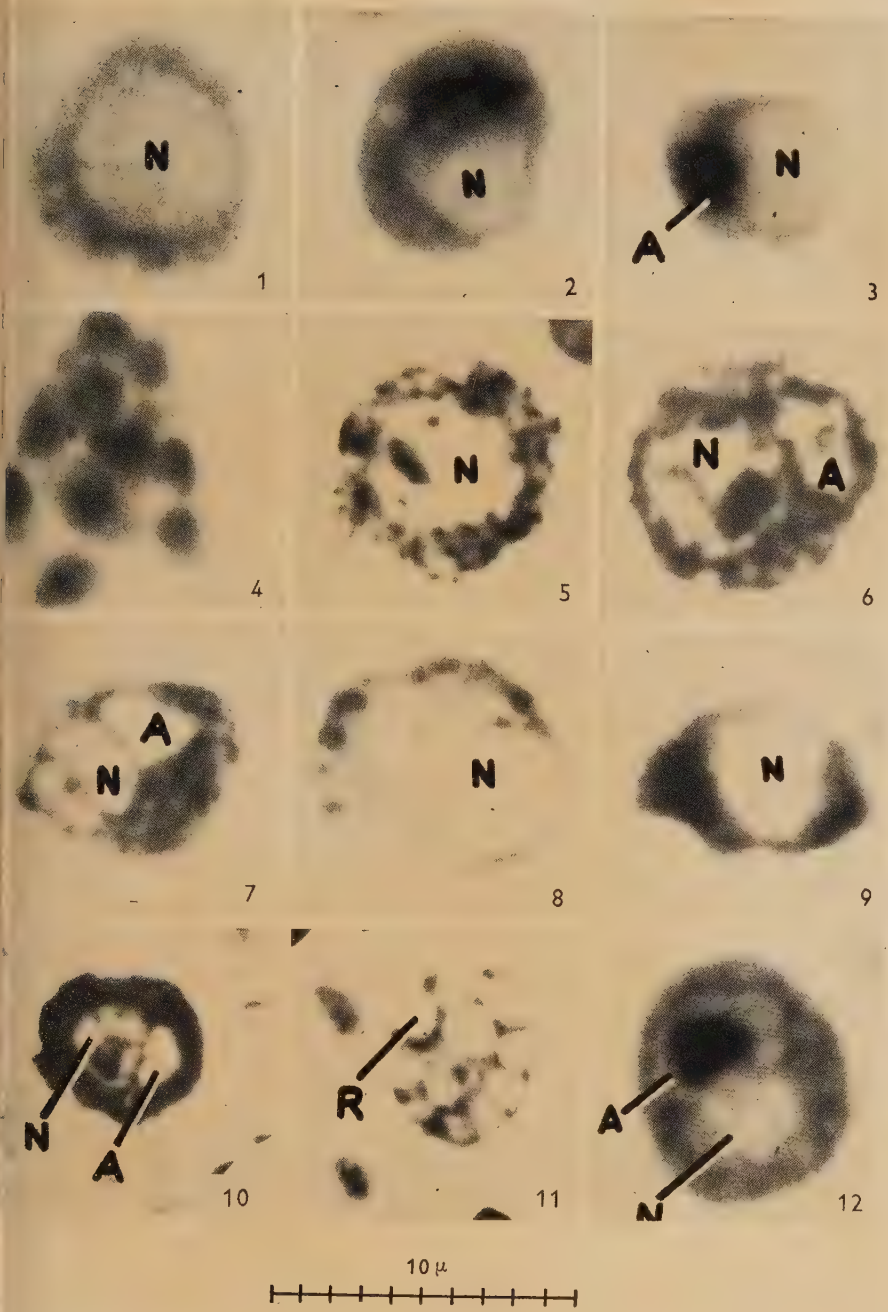
N, nucleus; *A*, area next to the nucleus; *R*, Russell Body.

Figs. 1–4. Imprints colored with Schiff's reagent after oxidation with periodic acid (carbohydrate). *Fig. 1*, blast cell. *Fig. 2*, proplasmacyte. *Fig. 3*, plasmacyte. *Fig. 4*, a cluster of Russell Bodies in which the colored Russell Bodies are separated by uncolored partitions.

Figs. 5–7. Imprints colored with Azure B (nucleic acids). *Fig. 5*, blast Cell, phase contrast optics. *Fig. 6*, proplasmacyte, note the heterogeneity of the area (*A*) next to the nucleus (*N*). *Fig. 7*, plasmacyte.

Figs. 8–11. Sections colored with Brom Phenol Blue (protein). *Fig. 8*, blast Cell; note the colored granules and contrast them with the Russell Bodies of Fig. 11, which appear in the more mature plasmacytes. *Fig. 9*, proplasmacyte. *Fig. 10*, plasmacyte, phase contrast optics. No. 29 red filter. *Fig. 11*, a cluster of Russell Bodies in which the uncolored Russell Bodies are separated by colored partitions.

Fig. 12. Imprint colored with Schiff's reagent after oxidation with periodic acid (carbohydrate). The area (*A*) next to the nucleus (*N*) consists of a central cluster of colored bodies surrounded by a layer of uncolored bodies.


N
1
N
2
A
N
3
4
N
5
N
A
6
A
N
7
N
8
N
9
N
A
10
R
11
A
N
12
10 μ

plasmacytes respectively. The strongest reaction occurred in an area (*A*) next to the nucleus (*N*) of the plasmacyte. Fig. 4 illustrates the intense reaction of Russell Bodies. Since the developmental sequence of plasma cells is known to procede from blast cell to proplasmacyte to plasmacyte and Russell Bodies [28], the figures suggest that detectable carbohydrate first appears throughout the cytoplasm and then becomes concentrated in the area next to the nucleus and in the Russell Bodies.

The staining of plasma cells with the Azure B method for nucleic acids has not been reported previously. There is some question about the specificity of the technique, but it is certainly more specific than the simple staining techniques previously applied [19]. Fig. 5 illustrates the appearance of fine fibrils and granules in blast cells. Fig. 6 illustrates the coarse cytoplasm and the heterogenous area (*A*) next to the nucleus (*N*) of some proplasmacytes. Fig. 7 illustrates the appearance of mature plasmacytes. The figures indicate that most of the nucleic acid of the cytoplasm is localized in microscopic fibrils and granules after calcium formol fixation. The fibrils probably represent artefactual aggregations of the submicroscopic lamellae previously demonstrated in the cytoplasm of plasma cells [7].

The staining of plasma cells with the Brom phenol blue method for protein has not been reported previously. The method is reported to be a specific and sensitive method for protein [29]. Fig. 8 illustrates the appearance of granules in blast cells. Fig. 9 illustrates the appearance of proplasmacytes in thin sections. Fig. 10 illustrates the relative paucity of protein in the area (*A*) next to the nucleus (*N*) and the abundance of protein in the rest of the cytoplasm of plasmacytes. Fig. 11 shows a mass of Russell Bodies (*R*), which do not stain strongly, divided by partitions which do. The figures indicate that plasma cells contain large amounts of protein, but that the area next to the nucleus, and the Russell Bodies, contain relatively little protein. (See, however, [14, 47] for the opinion that the Russell Bodies represent retained protein.)

DISCUSSION

It appears that the cells which produce large amounts of antibody and contain large amounts of nucleic acid and protein also contain large amounts of carbohydrate which is resistant to diastase digestion and to extraction with organic solvents. However, it should be noted that the diastase digestion for glycogen is supposed to work best for alcohol-fixed materials and alcohol fixation was avoided here because of its morphological effects, although these are not as severe as those produced by the Bouin's referred to below. The

tance to extraction with organic solvents varied. Much of the diffuse plasmic material, some of the material next to the nucleus, and little if of the Russell Body material was extracted in 24 hours by 60°C pyridine. results of using a modified Bouin's fixation and pyridine extraction od for complete removal of phospholipids [2] were even more difficult terpret. Fixation with mercuric chloride of pH 5.5 followed by prolonged action with water and acetone gave results indistinguishable from the l calcium formol fixed materials, which had been passed through the nol series, and this suggests that the Schiff reaction was not due to plas- We suggest that a lipoidal carbohydrate (1) is closely associated with ytoplasmic nucleoprotein, separates from the nucleoprotein in the course evelopment, and aggregates in the area next to the nucleus and in the ell Bodies and that it (2) is not composed to any appreciable extent of aldehydes. We are unable to make any elucidating suggestions as to the material may be, but it seems to undergo a progressive change in bility as it segregates in the area next to the nucleus and in the Russell es.

ne most distinctive morphological feature of plasma cells is the Russell y. This spherical to oval body may reach 10 μ in diameter and occurs y or in masses, in any one of which the bodies may be of similar size 11). Figures of such bodies were published in 1885 [13], their affinity for nic dyes (acidophilia) was described in 1898 [13], and the presence of ohydrate in them was established in 1949 [37]. The bodies usually ar bright and clear in unstained preparations and they are common eas which contain many plasma cells.

1890 [13], some acidophilic bodies which surrounded tumors were ribed as a characteristic organism of cancer. Subsequently it was [13] onstrated that such bodies are characteristic of plasma cells, which may ound around tumors as well as elsewhere. Since then these bodies have lly been identified by their acidophilia and their association with plasma l. This identification has been used by the clinician, pathologist, anato- and hematologist to define Russell Bodies. Therefore a body which is t and clear or acidophilic or contains non-metachromatic and relatively luble carbohydrate, other than glycogen, is a Russell Body in a plasma but is not a Russell Body in another cell. This practice has helped create mpression that plasma cells contain a body which has a unique signifi- e.

ven before Russell Bodies were associated with plasma cells it was sug- d that the acidophilic bodies are agents of cancer, parasites, phagocytized

erythrocytes, degeneration products of mitochondria, and degeneration products of mast cells [13]. Subsequent to their association with plasma cells it was suggested that the bodies are derived from the basophilic areas of the cytoplasm and from the myelin of the basophilic areas and are related to amyloid [13]. Of the last three suggestions the first is based on the fact that the bodies occur throughout the basophilic areas. The second is based on the brightness of myelin figures. The third suggestion is based on the occurrence of many plasma cells around amyloid deposits and the similarity of the staining reactions of Russell Bodies and amyloid. Some of the early suggestions have been repeated and other suggestions have been made. Since not all the clear bodies of plasma cells are acidophilic or contain demonstrable carbohydrate and not all of the bodies of plasma cells are clear, a great many different suggestions are possible. Crystals [24], presumably protein, which may or may not stain in the same way as the bodies, may occur in pathological material. These facts suggest to us that the Russell Body is not an entity essential to any specific function of the plasma cell, but is instead an aspecific expression of some process which is carried to an extreme by plasma cells. The variations suggest that some subsidiary process or processes may supersede the presumed principal process of plasma cells when the stimulus is prolonged as in the use of large amounts of antigen [42], adjuvants, or repeated injections of live bacteria [47]. The strong periodic acid–Schiff positive reaction of the Russell Bodies further suggests that one such subsidiary process is the synthesis of complex carbohydrate or of a lipid which is readily oxidized by periodic acid to an aldehyde.

The area next to the nucleus is usually referred to as the "Golgi Zone" and the spheres within it are sometimes referred to as "Golgi Bodies". In the plasma cell some spheres of this zone are periodic acid–Schiff positive and resemble the Russell Bodies. In other cells the spheres or "Golgi Bodies" have been reported to contain a periodic acid–Schiff positive material which is extractable by lipid solvents under some conditions [10]. This suggests that the Russell Bodies may tentatively be regarded as enlarged "Golgi Bodies", to rephrase an old suggestion [12]. It should be emphasized that not all of the non-basophilic area next to the nucleus of plasma cells is occupied by periodic acid–Schiff positive material, as shown in Fig. 12. Thus the "Golgi Zone" appears to contain spheres other than the ones which resemble Russell Bodies. This is consistent with the point of view that the area next to the nucleus or the "Golgi Zone" contains a variable mixture of discrete components and is not easily categorized as an entity [3, 10].

One of the difficulties in interpreting the significance of the area next to

nucleus of plasma cells derives from the obvious fact that the area is in contact with the nucleus and the basophilic cytoplasm. Therefore it is cult to decide whether the components of the area are derived directly the nucleus or the basophilic cytoplasm or both. One way to analyze situation might be to study the derivatives of the basal basophilic area lumnar epithelial cells in which the nucleus and the basal basophilic are separated and the "Golgi Zone" lies on the opposite side of the eus, as was suggested in 1921 as a result of studies of plasma cells [13]. e acidophilia of some derivatives of the basal basophilic area has been ibed [22] but this may or may not represent periodic acid–Schiff ive material. It should also be noted that the failure to detect a periodic Schiff reaction in the nucleus may not mean that little or no potentially ive material is present. Until some of these more general cytological ions are at least partially resolved it seems to us rather presumptuous sign "Golgi Bodies" or Russell Bodies anything more than an incidental in antibody, or even globulin, secretion as such. (But see White [47] an association of Russell Bodies with antibody secretion.)

rhaps any further study of the Russell Bodies should include alkaline phatase determinations since alkaline phosphatase activity has been ized in the "Golgi Zone" [44] and in other areas which are periodic acid–ff positive. We have no clue to the significance of this association, but o question the common interpretation that the carbohydrate is a poly-aharide. In all these cases it may be a lipoidal carbohydrate as indicated 54 for the "Golgi Bodies" of the head of the epididymis of the mouse [44].

SUMMARY

e synthesis of some serum globulins appears to be the principal anabolic ess of the plasma cells. If so, the syntheses of pentose nucleic acids and s must be quantitatively important subsidiary processes since the cyto-mic structures associated with protein synthesis contain high concentra-of pentose nucleic acid and lipid [26]. Our observations suggest that the nesis of complex carbohydrate may also be a quantitatively important idiary process with a function in the synthesis of at least some proteins. essential basis for this suggestion is (1) the pronounced occurrence of dic acid–Schiff positive material throughout the cytoplasm of young na cells, (2) its apparent progressive segregation from the nucleoprotein e cytoplasm, and (3) its pronounced retention in Russell Bodies beyond imes of peak plasma cell proliferation and maximum antibody release

[28]. In qualification of this suggestion it should be noted that (1) the positive material is only known to contain aldehyde groups after oxidation with periodic acid and that its identification as carbohydrate has not been demonstrated directly, (2) the equivalence of the positive material of different stages has not been demonstrated, (3) there is no apparent means of directly and readily distinguishing the late and presumably degenerative stages of the plasma cell from phagocytes, and (4) while the presence of a high concentration of pentose nucleic acid is indicative of an intensive synthesis of protein, high concentrations of intracellular lipid and carbohydrate are not evidence in themselves of intensive protein synthesis.

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ON THE CONNECTION BETWEEN THE SYNTHESIS OF RNA AND DNA IN THE TESTIS OF THE MOUSE

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THERE is a delay of one to eight days in the incorporation of labelled adenine ([8-^{14}C]-adenine) into the deoxyribonucleic acid (DNA) of the testis of the mouse [10]. It was shown by cell counts in autoradiographs of the corneal epithelium, that after a single injection of [^{14}C]-adenine labelling of the DNA of the highest number of cell nuclei occurs in this tissue within 24 hours. Similarly, in the small intestine, heavy labelling of the DNA of epithelial cells at the fundus of the glands can be observed one day after injection. In agreement with the findings of Leblond *et al.* [7], it was observed that two days after the injection the heavily labelled nuclei have moved half way up the gland, whilst labelling near their fundus is weaker at this time than after one day. In the testis, however, the percentage of spermatogonia whose DNA is labelled increases steadily from 12 per cent one day after the injection, to 63 per cent at ten days. This increase cannot be due to an increase in the number of labelled nuclei by cell division, since the number of grains per positive nucleus is constant. It was concluded that a DNA-precursor, either within the testis or elsewhere, becomes maximally labelled soon after injection, and is drawn upon by spermatogonia but not by cells of somatic tissues.

This paper reports further findings which provide additional information on the question of DNA synthesis by spermatogonia and the possible nature of the precursor.

MATERIALS AND METHODS

The methods used in this investigation are similar to those described in a previous communication [10], and are only briefly repeated here. Adult mice were injected intraperitoneally with 20–40 μC of [8-^{14}C]-adenine–HCl. Various organs were fixed for 1 hour in acetic acid alcohol (1:3), transferred to formol–saline for 24 hours,

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dded in paraffin wax, and autoradiographs of 5 μ sections were prepared by the ping film technique. Some slides were extracted with ribonuclease, deoxyri- clease or perchloric acid before applying the film; controls were kept under the conditions in 0.03 *M* veronal buffer at pH 7 or distilled water. Extraction with nes was carried out as previously reported; in addition, 10 per cent perchloric t 4°C with slow shaking was used to remove RNA from the sections; DNA and were removed with 5 per cent perchloric acid at 60°C.

stained preparations were viewed with phase contrast; others were stained with h's haematoxylin and eosin or methyl green-pyronin after photographic process- g[].

RESULTS

the testis, uptake of [8-^{14}C]-adenine, as judged by the autoradiographs in tracted slides, is relatively heavy in most of the non-dividing spermato- ox. and primary spermatocytes, weak in secondary spermatocytes and weak in later stages of spermiogenesis (Fig. 1). This pattern of localiza- s fairly constant up to six days after injection; later it changes consider- due to the movement of cells during their development, as would be ted from previous work [5, 10].

er extraction of RNA with cold perchloric acid (Figs. 2 and 3) or uclease, only the nuclei of spermatogonia are labelled; the number of ed spermatogonia in slides thus extracted increases steadily from 0–12 ent at 1 day to 50–60 per cent at 6 days, in agreement with previous s [10]. The residual bodies in the testis which were shown to consist A [4] do not stain with methyl green-pyronin after treatment with cold loric acid; other changes in stainability, such as weaker uptake of in the cytoplasm, confirm the efficacy of cold perchloric acid in ving RNA.

ly traces of labelled material remained in the sections after extraction ith hot perchloric acid (Fig. 4).

an be concluded that the nuclear autoradiographs observed after extrac- ith cold perchloric acid or ribonuclease are due to labelling of the DNA.

DISCUSSION

mparison of these observations on autoradiographs of unextracted and ted sections lead to the conclusion that [8-^{14}C]-adenine is fairly quickly porated into the RNA of spermatogonia and that it is utilized for DNA esis when a cell enters into the appropriate stage in its cycle. This ation of adenine incorporated into RNA might either proceed via a

breakdown of RNA followed by incorporation into DNA or the RNA might be a precursor for DNA. It seems unlikely that the adenine which is incorporated into DNA is derived from breakdown of RNA since there is no indication of greatly reduced amounts of RNA in tubules where the spermatogonia have synthesized DNA. It would therefore have to be assumed that only a limited quantity of RNA in the nucleus is broken down and some or all components parts re-utilized during DNA synthesis, an assumption which is almost indistinguishable from the second possibility, which is also supported by the results of previous cytochemical [1, 2, 3] and biochemical work [6, 11, 12, 13].

The observations seem best fitted by the assumption that RNA acts as a precursor for DNA in somatic tissues and in the testis but that the time lag between RNA synthesis and DNA synthesis is much longer in the testis than in the corneal epithelium and the small intestine. Some delay in the incorporation of labelled adenine into DNA has recently been found in other tissues [9] and the findings reported here should not be regarded as a difference between testis and all somatic tissues.

SUMMARY

The nucleic acids of various tissues were labelled by i.p. injection of [8-^{14}C]-adenine and autoradiographs of 5 μ sections were prepared. It has previously been reported that spermotogonia synthesize labelled DNA for a period of up to eight days after a single injection whilst no additional labelling of DNA takes place later than one day in the corneal epithelium and small intestine.

Observations on autoradiograhs of sections of mouse testes containing labelled RNA and DNA show that labelled adenine is incorporated into the RNA of most non-dividing spermotogonia within one day after injection. It is concluded that the observations can be explained if it is assumed that

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Autoradiographs of mouse testis after injection of [8-^{14}C]-adenine. Exposure time 55 days.
Fig. 1. Two days after injection. Stained with methyl green–pyronin. No extraction. The autoradiograph is heavy over the layer of spermatogonia and becomes progressively weaker towards the lumen of the tubules.
Fig. 2. Two days after injection. Extracted for 18 hours with 10 per cent perchloric acid at 4°C. Stained with methyl green–pyronin. Due to the extraction, uptake of pyronin and the autoradiograph are weaker, especially over spermatocytes and spermatids.
Fig. 3. One day after injection. Extracted for 18 hours with 10 per cent perchloric acid at 4°C. Stained with Ehrlich's haematoxylin and eosin. Note the nuclear labelling of some spermatogonia.
Fig. 4. Two days after injection. Extracted for 30 mins. with 5 per cent perchloric acid at 60°C. Stained with methyl green–pyronin. The extraction with hot perchloric acid has removed practically all of the compounds containing ^{14}C.

RNA acts as a precursor for DNA, as originally suggested by Brachet. The difference between testis and somatic tissues in the timing of the uptake of adenine into DNA indicates a different time-lag in the conversion of RNA to DNA.

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THE ACROSOMAL REACTION OF SPERMATOZOA DURING FERTILIZATION OR TREATMENT WITH EGG WATER

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The acrosome of several sea urchin species has been shown by Dan [7], by both phase contrast and electron microscopy, to respond to certain stimuli in a peculiar manner. Part of the acrosome disperses almost immediately, leaving a short filament protruding from the apex of the sperm which remains essentially unchanged thereafter. The stimuli used were egg water (i.e., sea water that has been in contact with sea urchin eggs for some time), alkaline sea-water (pH 9.2) or contact of the spermatozoa with glass or collodion. Similar acrosomal reactions have since been demonstrated in spermatozoa of starfish [8, 15], bivalve molluscs [10], some worms [5], some holothurians [5] and the frog *Discoglossus pictus* [11].

In some animal groups an acrosomal filament extends anteriorly in the unmodulated spermatozoon. This is true for the spermatozoon of the lamprey, *Lampetra fluviatilis* (*Petromyzon f.*) [4, 20], the chiton spermatozoon [12, 18, 19, 22] and spermatozoa of certain *Solenogastres* [12].

The functional significance of the acrosome remains largely unknown. Tyler, Collier and Dan [28] demonstrated that the acrosomal material of *Mytilus edulis* (common mussel) spermatozoon liberated by egg water treatment contains a lysin capable of dissolving the vitelline membrane of the egg. Some years earlier Tyler [27], on the basis of a study with a variety of techniques including electron microscopy, postulated the existence of such a lysin in the acrosome of the spermatozoon of another mollusc, *Arbacia punctulata*. Dan [8] believes that the acrosomal filament attaches the spermatozoon to the vitelline membrane of the egg, and stimulates the egg cortex to activity in the spermatozoon by this filament. The entrance of the acrosomal filament into the egg was followed *in vivo* by Colwin and Colwin [6] in the sea cucumber *Holothuria atra* and the starfish *Asterias amurensis*. Both this paper and that of Dan [8] review the different interpretations of sperm–egg interconnecting filaments in fertilization described by earlier investigators.

The appearance of the acrosomal filament during fertilization has not

yet been studied with the electron microscope. The present study describes the acrosomal reaction in normal fertilization and compares this reaction to the acrosomal reaction after egg water treatment. The use of ultra-thin sectioning technique after Sjöstrand [24, 25] reveals details not seen in the whole mounts. The fine structure of the untreated sea urchin spermatozoon has been described in an earlier paper [1]. The substance of the present paper was presented at the First European Regional Conference on Electron Microscopy held in Stockholm in September, 1956 [2].

MATERIAL AND METHODS

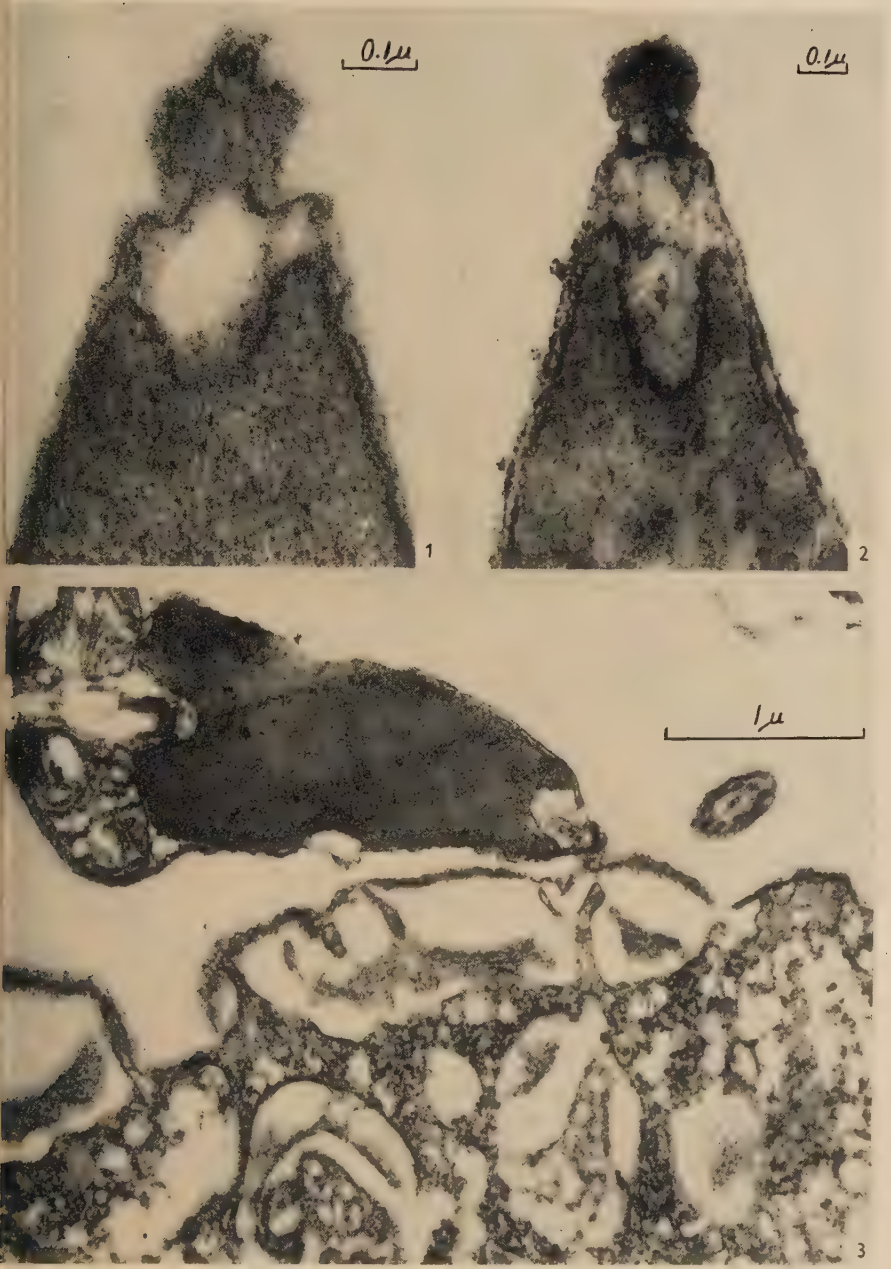
The following sea urchin species were used in this investigation: *Strongylocentrotus droebachiensis* (collected at the Trondheim Biological Station), *Echinus esculentus*, *Psammechinus miliaris*, *Brissopsis lyrifera*, *Echinocardium cordatum*, and *Echinocardium flavescens* (all collected on the Swedish west coast). The spermatozoa were inspected with oil immersion phase contrast microscopy, using a Cooke microscope with a 95 × objective lens, with a NA of 1.30. In the fertilization experiments eggs were inseminated with sperm suspensions containing 10^6–10^7 spermatozoa per ml, and samples of the eggs were fixed 2 to 3½ minutes after sperm addition. The eggs were kept at the temperature of their normal environment; eggs that showed a somewhat slow fertilization rate were preferred in order that a closely graded series would be available for study. The subsequent development of the fertile eggs were quite normal. Fixation was performed in 1 to 3 per cent osmium tetroxide in sea water, and dehydration and embedding in methacrylate by a slight modification of the technique of Newman, Borysko and Swerdlow [16]. Sections from the Sjöstrand microtome [23] were mounted from a liquid surface [13] on coverglasses for phase microscopy. Adjacent sections were mounted on specimen grids covered with a thin formvar film. They were examined in an RCA EMU 2c electron microscope with compensated standard objective pole piece, standard objective aperture, three apertures in the projector lens and a reduced condensor lens aperture. The technique for preparing these matched specimens is described by Swanbeck [26].

In other studies, spermatozoa of the lamprey, *Lampetra fluviatilis*, were used. The spermatozoa from male animals were stripped directly into the fixative, thus avoiding contact with fresh water. The fixatives used were 1 per cent osmium tetroxide in Tyrode's solution or 5 per cent formaldehyde in Tyrode's solution. The spermatozoa were mounted on grids, washed in distilled water and examined in the electron microscope as whole mounts, or embedded and examined in ultra-thin sections according to the above methods.

Fig. 1. *Echinus esculentus* spermatozoon, which in the immediate vicinity of the egg has undergone acrosomal reaction. Magnification × 100,000.

Fig. 2. *Psammechinus miliaris* egg water treated spermatozoon. The acrosomal globule is expelled. Magnification × 68,000.

Fig. 3. *Psammechinus miliaris*. A spermatozoon is in contact with the egg surface and the acrosomal globule is almost dissolved. A few cortical granules in the egg cortex have exploded. Magnification × 27,000.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OBSERVATIONS

Egg Water Treated Sea Urchin Spermatozoa

Studies on living material.—The most conspicuous changes revealed by this technique occurred in the acrosomal region, the middle piece and the tail. These transformations were not uniform throughout the samples examined, and usually the majority of the spermatozoa showed no visible change.

The following changes were observed in the acrosomal region: (*a*) The acrosomal globule was sometimes dissolved, giving a more blunt appearance to the head. (*b*) In some cases, especially in the *Echinocardium cordatum* spermatozoon, when the acrosomal stalk [1] is lost, the anterior part of the head was hollow, resembling an open mouth. (*c*) In other spermatozoa the acrosomal globule was slightly displaced and a less dense material separated it from the anterior part of the nucleus. This condition persisted in many spermatozoa for several minutes under constant observation. (*d*) In still other spermatozoa a very thin filament extended anteriorly from the acrosome, straight or slightly curved, usually varying in length from approximately 0.5 μ to 3 μ and occasionally as long as 12 μ. (*e*) Many spermatozoa showed apical threads exceeding 20 μ in length. On these there were no tails in the normal position, suggesting that the tails were bent forward, projecting from the tip as a fibril resembling the acrosomal filament.

In many egg water treated spermatozoa the middle piece, in contrast to its normal position at the base, lay lateral to the nucleus and was frequently enlarged. All the acrosomal changes described above were similar in spermatozoa from the five sea urchin species examined.

These alterations could be seen even in untreated spermatozoa but only rarely. An acrosomal reaction was observed occasionally in spermatozoa

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Fig. 4. *Strongylocentrotus droebachiensis* spermatozoon with the acrosomal globule outside the cell membrane. Magnification × 60,000.

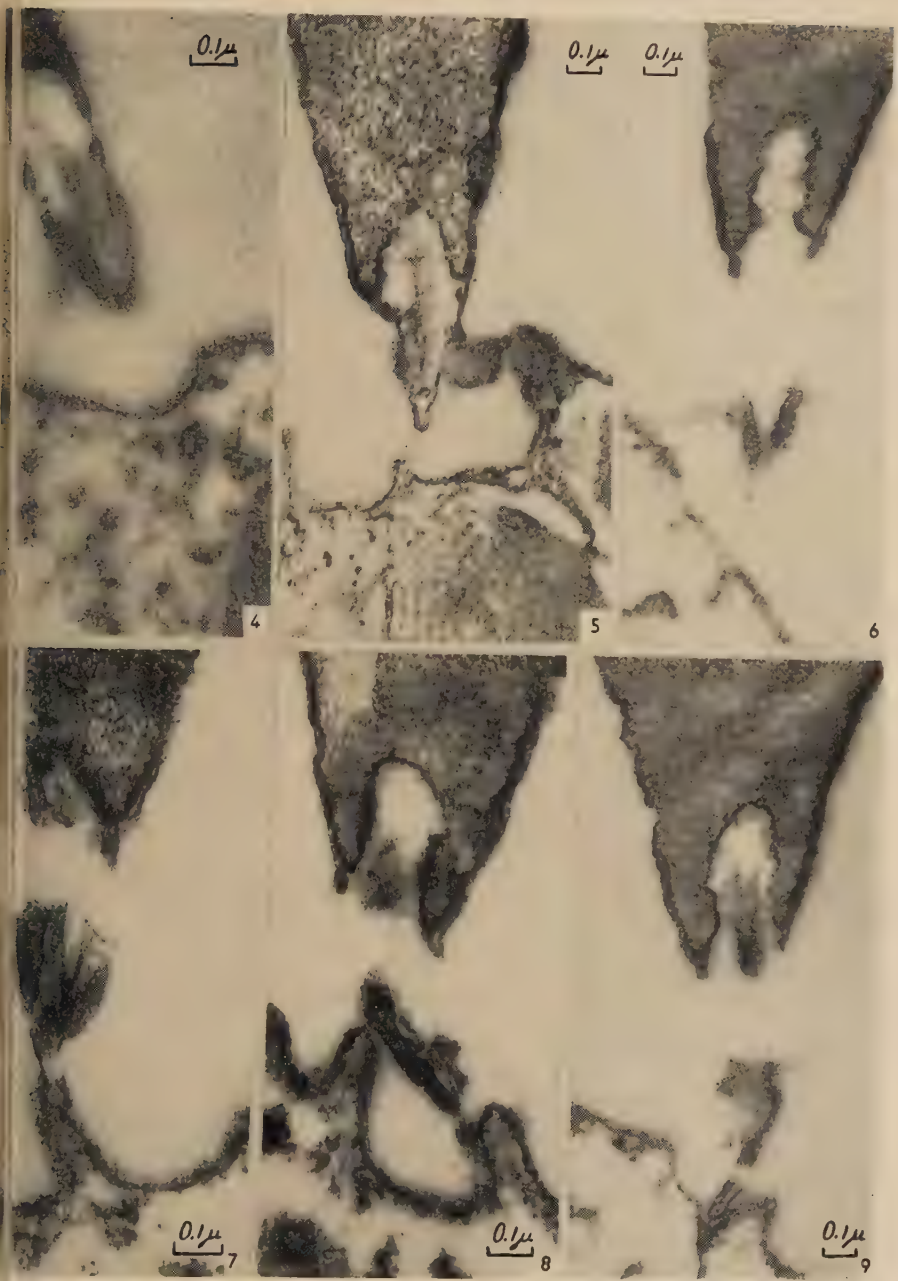
Fig. 5. *Strongylocentrotus droebachiensis* spermatozoon in contact with the egg surface. The expelled acrosomal globule is to the right of the cell membrane which does not seem to be interrupted. The material within the nuclear indentation is clearly fibrillar. Magnification × 48,000.

Fig. 6. *Strongylocentrotus droebachiensis* spermatozoon in contact with the egg surface. The expelled acrosomal globule forms a ring around the cell membrane and is separated from the nucleus. Magnification × 45,000.

Fig. 7. *Strongylocentrotus droebachiensis* spermatozoon with a filament attached to an egg process and surrounded by the acrosomal globule. Magnification × 65,000.

Fig. 8. *Strongylocentrotus droebachiensis* spermatozoon with a transformed acrosomal globule and to the right of this the acrosomal filament in contact with the egg surface. Magnification × 60,000.

Fig. 9. *Strongylocentrotus droebachiensis*. The acrosomal filament between the nuclear indentation and the egg surface is broken into three parts. Magnification × 47,000.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adhering to the coverglass, but in other preparations many hundreds o spermatozoa attached to the coverglass were inspected, all having the acroso mal globule intact.

Studies on fixed and sectioned material.—Comparison under the phas microscope of living spermatozoa with those fixed in osmium reveals n significant alteration of morphology, except that the acrosomal filaments ar much shorter in the fixed material. Figs. 11, 12 and 13 and Figs. 2 and 1 show comparable sections from the same spermatozoa, demonstrating th appearance under phase contrast and with electron microscope of fixed an sectioned material. It can be seen that the acrosomal globule lay some distanc away from the apical end of the nucleus but retained its position near th cell membrane. The acrosomal globule was, however, outside the cell mem brane, although the cell membrane appeared not to be ruptured at an point. A few vesiculate bodies were seen in the apical nuclear indentatio

The appearance of the acrosomal globule outside the cell membrane wa a constant feature in spermatozoa that had undergone acrosomal reactio Vesicles in the nuclear indentation approximately 0.06 μ in diameter we seen only occasionally. There was a distinctly fibrillar appearance to th material within the nuclear indentation. In many spermatozoa the anteri end of the head was open, suggesting that the acrosomal globule carrie with it a segment of cell membrane. The mean depth of the apical nucle indentation was within the range found in unreacted spermatozoa. Fo example, the mean value in normal *Strongylocentrotus* spermatozoa wa 0.43 ± 0.03 μ; for reacted spermatozoa 0.41 ± 0.02 μ. Comparable valu for *Psammechinus* spermatozoa were 0.23 ± 0.03 μ and 0.26 ± 0.03 μ respe tively. All values were calculated on the basis of 10 measurements.

The spermatozoon response to egg water treatment showed a wide vari tion within the acrosomal region as well as in other parts of the spermatozoo The cell membrane was often separated from the enclosed structures by a empty space and many of the mitochondrial double membranes of the midd piece had swollen to vesicles and had taken a position alongside the nucleu In some spermatozoa the tail filaments also were located along the nucle and within the cell membrane of the head (see also [1], Fig. 3). Anoth abnormality noted in the egg water treated material was the presence of tw sets of tail filaments within a common cell membrane.

Fig. 10. *Strongylocentrotus droebachiensis* spermatozoon. The expelled acrosomal globule togeth with a segment of the cell membrane and a filament lie near a process of the egg. Magnificati $\times 36{,}000$. The lower inset shows the expelled acrosome magnified to $\times 88{,}000$, and the upp inset shows the centriole magnified to $\times 73{,}000$.

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Sea Urchin Spermatozoa during Fertilization

e spermatozoa swarming around inseminated eggs showed some of the ges observed in egg water treated spermatozoa. Separated acrosomal les, acrosomal filaments and tails protruding from the anterior end of ead were seen. Whether the spermatozoon which started the reaction leading to fertilization had undergone any of these changes could not termined.

ced and sectioned material.—All the spermatozoa attached to the egg ce in the initial stages of fertilization showed the acrosomal reaction. me cases the reaction involved only a transfer of the acrosomal globule outside of the cell membrane, where it was easily recognized by its size homogenous density (Figs. 1, 4, 5). In other cases the cell membrane was ured, and the acrosomal globule, often ring-shaped with an adhering of cell membrane, had been expelled (Figs. 6, 7, 8, 9, 10). A distinct ent sometimes connected the nucleus with the globule (Figs. 7, 8, 9). e acrosomal globule of the untreated spermatozoon appeared dense osmium fixation, but non-dense in frozen dried material or after fixation ethyl mercury chloride according to the technique of Bahr and Mober- 3].

hough discontinuous filaments of greater lengths were seen, the maximum h of continuous acrosomal filaments was 0.7 μ. The absence of the long ents seen on living spermatozoa probably resulted from breakage or during osmium fixation. (Note the broken filaments in Fig. 9.) In the natozoa with expelled acrosomes the sea water seemed to come in direct ct with the nuclear membrane of the apical indentation. The rest of ell was still covered by the cell membrane. The gap, formed when the ome was expelled, between the disrupted cell membrane and the nuclear brane was bridged by an annular structure (Figs. 7, 8, 9) of unknown n, which possibly formed a barrier to the entrance of sea water to the cell plasm. The formation of this bridging annulus might be taken as an nce that the rupture of the cell membrane is the result of the acrosomal ion rather than the treatment during fixation and embedding, although atter possibility cannot be excluded.

1–13. *Psammechinus miliaris* egg water treated spermatozoa. Phase contrast microscopy sections showing successive stages in the separation of the acrosomal globule and a short mal filament. Magnification × 7,000.

1. *Psammechinus miliaris* egg water treated spermatozoa. Magnification × 30,000.

5. *Lampetra fluviatilis.* Whole mount electron micrograph of the central parts of a spermato- Magnification × 15,000.

The examined acrosomes in sperm–egg contacts were from 35 eggs o *S. droebachiensis*, 14 eggs of *E. esculentus*, and 17 eggs of *P. miliaris*.

The lamprey spermatozoa

Fig. 15 shows the central parts of a *Lampetra* spermatozoon. The thin filament extending from the anterior part of the head can be seen on the upper right part of this figure. Only a very small part of the filament is included in the figure, its total length being approximately 50 μ. The thickness of the filament is about 0.06 μ both in osmium and formaldehyde fixed samples. In contrast to the tail, it shows no clear fibrillar fine structure, but has a rather homogeneous appearance in whole mounts as well as in sections. This long filament is anchored deep in the nucleus, but there is no evidence that it is connected with the centriole, as Ballowitz [4] and Retzius [20] assumed.

DISCUSSION

The sperm acrosome of the sea urchin consists of two parts, an acrosomal globule and material within the apical nuclear indentation [1]. The present paper is an attempt to analyze the function in fertilization of the two parts. Freshly inseminated eggs have been used to which, at the time of fixation, several spermatozoa were attached. All examined spermatozoa in contact with the egg surface or in its vicinity exhibited an acrosomal reaction similar to that first described by Dan [7]. In the reaction the acrosomal globule was always expelled, but a filament could not always be observed.

The excretion of the acrosomal globule was first noted by Popa [17], who also stated that this process made the sperm tip sticky. Rothschild and Tyler [22] studying whole mounts of spermatozoa of certain marine animals postulated that the acrosomal globule formed the acrosomal filament by unrolling and that this process was possibly reversible. In a later study Rothschild [21] using sections examined with the electron microscope, suggested that the nuclear indentation behaves like an invaginated structure, which is everted in the acrosomal reaction. Neither of these explanations is consistent with our findings. The acrosomal globule and the acrosomal filament may co-exist, and the material within the nuclear invagination, rather than the acrosomal globule, seems to be the source of the filament. The dimensions of the nuclear invagination are practically the same before and after the acrosomal reaction.

The idea that the two separate entities in the acrosome act differently in fertilization [7, 1] is supported by our findings. The role of the filament of

tarfish spermatozoon according to Dan [8] is to penetrate the jelly coat vitelline membrane and to stimulate the egg cortex. The egg cytoplasm draws in the filament with the attached spermatozoon. As early as 1905 witz [4] expressed a similar opinion on the function in fertilization e long acrosomal filament "Kopfborste" of the lamprey spermatozoon: ses lange Gebilde muss ... weit früher mit seiner Spitze das Eiproto- ma des aktiven Poles treffen und in dasselbe eindringen, als die Kopf- die äussere Eifläche erreicht. Das agile Eiprotoplasma hat also durch eindringende Kopfborste den Anreiz für seine Bewegungsphänomene n längst erhalten ..."

was not until very recently that the entrance of the acrosomal filament the egg was followed *in vivo*. These observations were performed by in and Colwin [6] with the light microscope on holothurian and starfish ization, and their photographs clearly demonstrate that the slender ent did not change its dimensions or alter its position on the sperm head after the head itself had entered the egg. It is therefore unlikely that perm head is conducted to and into the egg by contraction of the acroso- ilament. Spermatozoa that were attached to the eggs but did not subse- tly enter them had filaments similar to those of the fertilizing spermato- oa.

seems probable that the same conditions prevail during fertilization in rchin as in the two echinoderm classes investigated by Colwin and Col- The acrosomal filament is clearly shown in the present study, and the hat it is not always seen may be due to unsatisfactory fixation.

ny investigations have been performed in search of substances in the natozoa that have a lytic effect on the egg surface. These substances have called sperm lysins but do not constitute a uniform group of compounds. study adds little information in solution of this question. No dissolution e vitelline membrane was found in the vicinity of the expelled acrosomal ule in the sections studied. As the vitelline membrane appeared well ed only when sectioned perpendicularly, reliable knowledge of "sperm s" action on the vitelline membrane can only be obtained from sufficient ons of sperm–egg contacts to allow a statistical treatment of the material. e reaction of the egg water treated spermatozoon was found to be more ble than that of the spermatozoon attached to the egg in the initial phase tilization. The first effect of egg water treatment seems in most cases to change in the properties of the cell membrane. This is evidenced e.g. wollen and burst cells, tails within the cell membrane alongside the us, and two sets of tail filaments within common cell membrane.

The effect of egg water on sperm morphology may now be compared with the changes induced in spermatozoa during fertilization. Egg water treatment produced a number of deformed and burst cells that may hardly be capable of fertilization, yet more than half of the spermatozoa in the same samples exhibited no visible change. Hagström [14] has shown that more spermatozoa are needed to get the same fertilization percentage in eggs with jelly coats than in jelly free eggs, a proof that the jelly coat surrounding the sea urchin eggs to a certain extent acts to inhibit fertilization. The life span of the spermatozoa is, however, longer in egg water than in sea water, which seems to be a non-specific effect [29]. Thus, while the jelly coat has an imparing effect on some spermatozoa, others preserve their mobility and fertilizing ability longer than in sea water. Purely morphological studies and fertilization experiments prove that not all spermatozoa react similarly to egg water treatment.

Until further pictures are obtained—and pictures of the actual sperm penetration—it is not possible to know how the acrosomal region acts in fertilization. We have no evidence of an alteration of the vitelline membrane of the egg in proximity with the ejected acrosomal globule. The fact that every spermatozoon at the egg surface has undergone acrosomal reaction and that the acrosomal globule has taken a position nearest the egg is, however, consistent with a hypothesis that the acrosomal globule contains substances active in sperm penetration. It is plausible that the acrosomal filament is an accessory means of forwarding the active substances of the acrosomal globule to the egg surface. Because of their exposed position after acrosomal reaction these substances may be inactivated within a limited time.

SUMMARY

The transformation of the acrosomal region during fertilization or by treatment with egg water has been studied by electron microscopy of ultra-thin sections prepared according to the technique of Sjöstrand and by phase contrast microscopy.

1. Spermatozoa fixed 2 to 3½ minutes after addition to the eggs had all undergone acrosomal reaction.

2. In most reacted spermatozoa the less osmiophilic material was oriented lengthwise to a filament which pushed the cell membrane to an apex. The excreted acrosomal globule was always outside the cell membrane and often formed a ring around this apex.

3. In many reacted spermatozoa the cell membrane was broken and a filament projected anteriorly from the nucleus. This filament was presumably formed from the less osmiophilic acrosomal material.

Essentially the same changes could be seen in the living state with oil mersion phase contrast microscopy. The acrosomal filament seen *in vivo* occasionally as long as 12 μ, whereas the fixed and sectioned ones never exceeded 0.7 μ.

The response of the spermatozoon to egg water treatment showed a variation within the acrosomal region as well as in other parts of the spermatozoon. Most transformations seemed primarily to involve the properties of the cell membrane. More than half the spermatozoa in the egg water treated samples exhibited no visible changes.

The 50 μ long acrosomal filament of the lamprey spermatozoon was present in freshly ejaculated sperm and showed a rather homogeneous ultrastructure.

The possible roles of the two parts of the acrosome are discussed.

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PROTEIN PRODUCTION BY THE RAT PANCREAS

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During a series of studies on the protein synthesis occurring in the pancreas [10, 4, 7] it became desirable to estimate the protein production of this organ in the rat and the amount of protein secreted by each pancreatic acinar cell under different conditions.

The pancreas is a favorable subject to study protein synthesis, for it secretes proteins with intensity and this activity can be controlled by physiological and pharmacological methods. Furthermore, previous studies have been performed on this gland furnishing a number of data that considerably simplify the subject (for a review cf. [6]).

We present in this paper the results obtained studying the protein production in the pancreas of rats with chronic pancreatic fistula, and in anesthetized rats with or without secretory stimulation. Furthermore the number of cells in the rat pancreas as well as the amount of protein per pancreatic cell were estimated.

MATERIAL AND METHODS

Adult male Wistar rats weighing from 200 to 300 g were used. Desoxyribonucleic acid (DNA) determinations were performed according to the method described by Brody [1]. The number of cells per pancreas was calculated dividing the total amount of DNA of this organ by the amount of this substance present in each pancreatic cell nucleus. For the amount of DNA per nucleus the result of Thomson, Heagy, Hutchison and Davidson [12], i.e. 7.38×10^{-6} μg, was used. This result is very close to those obtained for different rat organs by other authors [14, 15].

The rat pancreas presents a relatively homogenous nuclear population in regard to their DNA content [13], a fact that permits calculations without too great an error.

The total protein content of the rat pancreas was measured by Kjeldahl's method and the amount of protein per cell was obtained dividing these results by the number of cells per pancreas.

The protein secreted in the pancreatic juice was assayed with the method described by Lowry, Rosebrough, Farr and Randall [9] after a previous check with Kjeldahl's method.

The pancreatic juice of anesthetized animals (groups 1 and 2) was collected through a fine polyethylene cannula inserted in the common biliary and pancreatic duct after

rming a ligature of the biliary duct 5 mm below its emergence from the liver. thesia was obtained by intraperitoneal injection of 8.0 mg of Numal (Roche) per ; body weight. Secretion was stimulated in group 2 by endovenous injection of xture containing 1.0 μg carbaminoylcholine plus 0.7 units of secretin (Lilly) 2 ml saline per rat. Rats were fasted during 18 hours previously to eliminate ossible stimulation by food. A detailed account of this procedure was published here [7]. The results of these two groups refer to the amount of protein secreted g the first hour of experiment.

the animals with chronic fistula (group 3) the twenty-four hour secretion product collected and expressed on an hourly basis. Juice was collected every day during to five days after the operation. The chronic fistula was performed according e method described by Colwell [2]. This procedure in the rat promotes a relatively deterioration of the animal's health and death in 3 to 5 days. The results ob- d are variable and far from physiological, and should therefore be considered ch.

e percentage of acinar cells in the pancreas was estimated by counting 1300 i in 24 different random microscopic fields of histological sections obtained from reases of four rats.

RESULTS AND DISCUSSION

ne results obtained are expressed in Tables I and II and their study ests the following remarks:

o comparison can be made between the rate of pancreatic juice flow animals with chronic fistula and those anesthetized, with and without ulus, for in these last groups the animals were fasted 18 hours previously. anesthetized non stimulated animals produced the smallest amount of

TABLE I

Average composition of the normal rat pancreas (5 animals).

weight	273.0 $\pm$ 25.3 g	Percentage of acinar cells in the pancreas	77.5 per cent
eas weight	854.0 $\pm$ 97.3 mg	Number of acinar cells in the pancreas	1.63×10^8
in amount per ncreas	180.0 $\pm$ 9.5 mg	Protein amount per acinar cell	8.5×10^{-4} μg
amount per ncreas	1545.40 $\pm$ 89.4 μg	RNA amount per acinar cell [b]	39.1×10^{-6} μg
amount per ncreatic cell [a]	7.38×10^{-6} μg		
er of cells per ncreas	2.11×10^8		

ata from Thomson, Heagy, Hutchison and Davidson [12].
alculated from data presented by Leslie [8].

TABLE II

Protein production in the rat pancreas.

Condition ...	Anesthetized (12 rats)	Anesthetized plus stimulus [b] (8 rats)	Chronic fistula (3 rats) [c]
Juice volume [a]	15.9 ± 4.4 μl	62.7 ± 24.9 μl	50.0 μl
Protein concentration	5.18 ± 1.7 per cent	3.4 ± 1.2 per cent	2.7 per cent
Total protein production per pancreas per hour [a]	0.823 mg	2.136 mg	1.35 mg
Protein secreted by one acinar cell per hour	0.50×10^{-5} μg	1.310×10^{-5} μg	0.828×10^{-5} μg
Percentage of the acinar cell protein secreted per hour	0.58 per cent	1.54 per cent	0.97 per cent

[a] Amount secreted in the first hour of experiment. In the chronic fistula group the hourly average of 24-hour samples.
[b] Carbaminoylcholine plus secretin.
[c] No standard deviation is presented due to the samll number of animals.

protein per hour, followed by the group with chronic fistula and the anesthetized stimulated animals.

Few data exist referring to the protein content of individual glandular cells. Our result of 8.5×10^{-4} μg protein per acinar cell is close to those calculated for the rat liver cells from data presented by Schneider [11] and Leslie [8], i.e. 6.2×10^{-4} μg and 7.2×10^{-4} μg respectively.

The extrusion of proteins due to cell secretion from the acinar cells oscillates between 0.58 to 1.54 per cent of its total protein content per hour, according to the group of animals studied. A gross calculation would suggest therefore that the amount of protein secreted daily by the rat pancreas lies somewhere around 23 to 37 per cent of its total protein content.

It is of interest to refer here to the data presented by Daly and Mirsky [3] and Hokin [5]. The former calculated that 20 per cent of the proteins of the acinar cells of the mouse pancreas are represented by the digestive enzymes, while the latter comes to the conclusion that the zymogen granules account for 40–50 per cent of the total nitrogen of the pancreas of a 24-hour fasted dog.

Our results suggest that in the interpretation of data referring to the incorporation of amino acids into the pancreas proteins, allowance should be made to the rate of incorporation into the proteins of the digestive enzymes secreted on one side, and into the proteins of the cell structure on the other.

is point of view is strengthened by the fact that quite different rates of poration of phenylalanine-3-C^{14} into the proteins of total homogenates those of secretory granules are observed (unpublished results of this atory).

SUMMARY

e protein production by the pancreas of rats with chronic pancreatic sa as well as in anesthetized animals with and without secretory stimula- o was studied.

e average weight, protein and DNA amounts, number of cells and per- ge of acinar cells per pancreas was determined.

om these data the amount of protein present in one acinar cell and the tity of protein secreted by each acinar cell were calculated.

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VOLUME CHANGES OF TISSUES IN PHYSIOLOGICAL FLUIDS DURING FIXATION IN OSMIUM TETROXIDE OR FORMALDEHYDE AND DURING SUBSEQUENT TREATMENT[1]

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In a recent paper [6] an analysis was presented of the changes of volume, weight and specific gravity of tissue specimens during histological fixation and embedding. The investigation procedure was based on the principle of Archimedes and was applied to fixation in pure alcohols, followed by clearing in xylene and infiltration with paraffin. It was deemed desirable to extend the investigation to other common fixatives. Those chosen were osmic acid and formaldehyde, each an outstanding fixative in the fields of electron and light microscopy, respectively. In the course of the experiments results were obtained which prompted a closer study of osmotic phenomena too.

Earlier literature contains numerous reports of attempts to elucidate these problems. Reviews have been presented by Rexed [32], Ambrosius [1], Wüstenfeld [40, 41], among others. The datas given are, however, not fully comparable, partly because they are based on a few experiments only and partly because the specimens have included special types such as sea urchin eggs [18], salamander cartilage [18], whole human brains [12, 19–27], cell nuclei [1, 9], erythrocytes [34, 35], proteins [34, 35], etc.

Few reliable data are available for the evaluation of shrinkage or swelling during osmium fixation. In 1 per cent osmium tetroxide egg cells from the cow showed an increase of volume varying from 1.5 to 25 per cent [22]. Cells from tissue cultures after fixation in 2 per cent osmium tetroxide suffered no change in volume [38]. The diameter of nerves increased with about 2–7 per cent after osmium fixation [8, 20].

Volume or weight changes after fixation in formaldehyde have been

[1] An abstract of these studies was presented before the First European Regional Conference on Electron Microscopy, Stockholm, 1956.

ed to a greater extent. Brain tissue, in various concentrations of this ve, showed a weight increase ranging from 3–40 per cent [12, 19, 23, For various organs from the rat and the mouse after completed fixation per cent formaldehyde a swelling of 3–20 per cent has been reported 3]. The swelling was followed by marked shrinkage after dehydration paraffin embedding. Pig embryos fixed in 4 per cent formaldehyde for ours showed an average increase in length of 5 per cent with subsequent kage during dehydration/embedding [31]. Trout embryos have been with similar results [26].

rlier work was criticized by Hertwig [18] as being unreliable for cyto- al purpose. He worked on salamander cartilage as well as on sea urchin and he too found a swelling after fixation in formaldehyde. Tarkhan sought to measure volume changes during formaldehyde fixation etc. experimental arrangements were such, however, that hardly any con- ons can be drawn from his results.

eight changes in specimens fixed in formaldehyde of various pH values been reported [16]. In 1 per cent neutral formaldehyde a weight in- e of 70 per cent was found. At pH 2 it amounted to 45 per cent and at 10 to 80 per cent. The weight increase could be reduced by adding um chloride and potassium bichromate to the fixative. Stowell [37] using direct method for studying volume changes of homogenous pieces of e during fixation, dehydration and embedding, found that fixation in er cent formalin gave rise to a volume increase of around 35 per cent. dehydration in alcohol, clearing in xylene and embedding in parawax, al shrinkage of 40–45 per cent was noted. Recently, Wüstenfeld [40, 41], a technique similar to ours [11], studied the effect of a 4 per cent formal- de solution on liver, muscle, spleen and kidney. He found an average ling of 11 per cent after 210 hours of fixation. This author reported mal swelling for liver after 4 hours, kidney after 17 hours and for le and spleen after 21 hours. These data, however, were based on one piece of tissue for each determination.

crocinematography in the phase contrast microscope has been used bserve the changes during fixation of tissue cultures. Planimetrical urements of cell areas showed an increase of about 10 per cent [17]. n extensive study Crawford and Barer [7] observed in the phase con- microscope the action of formaldehyde on isolated salamander sper- gonia and tissue culture snail amoebocytes. In 5 per cent formaldehyde found that specimens passed through a short period of shrinkage, a d of swelling, and lastly a period of secondary shrinkage. The first

stage varied with the concentration of the fixation fluid. Above 10 per cent formaldehyde, initial shrinkage was greater and was followed by little or no swelling. Addition of salt increased the initial shrinkage and reduced the subsequent swelling. In contrast to the meagre literature on osmium fixation a considerable number of articles on formalin fixation have thus been published. Summing up these papers, it may be said that fixation in formaldehyde causes swelling followed by a gradual decrease of volume, but the data presented vary greatly. Generally, only the final values after each step have been recorded, and therefore only in a few instances are data available on the progressive changes during the action of a single agent.

MATERIAL AND METHODS

Specimens were obtained from guinea pigs, killed by a blow on the head and exsanguination. In most experiments liver was preferred because of its obvious advantages (size, homogeneity, etc.). In some experiments other tissues were used as well for comparative purposes. Slices weighing 150–200 mg and measuring about $2 \times 10 \times 10$ mm were used. Measurements were always made on groups of at least five specimens each, and the experiments were repeated at least twice. The specimens were suspended by thin nylon threads and their weights in air and in the different fluids were recorded. From these measurements, the volumes, weights and specific gravities were computed. For further details of the procedure, the original paper [6] should be consulted. A method similar to ours has been proposed by Feneis [10].

Fixation, dehydration and infiltration with methacrylate (*n*-butylmethacrylate benzoylperoxide as a catalyst) were performed at two different, constant temperatures, viz., 0°C and 24°C. Specimens to be embedded in paraffin were cleared in xylene for 30 minutes at 24°C and were then infiltrated with Histowax (Mathesons Co., Norwood, U.S.A.) at 58°C overnight (around 16 hours). Analytical grade reagents were used, with the two exceptions given.

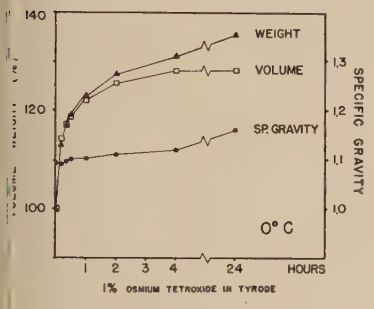
The dextran (Macrodex) used in this study was kindly supplied by Pharmacia Ltd., Uppsala, Sweden. It has a mean molecular weight of 80,000 and an intrinsic viscosity of 0.26.

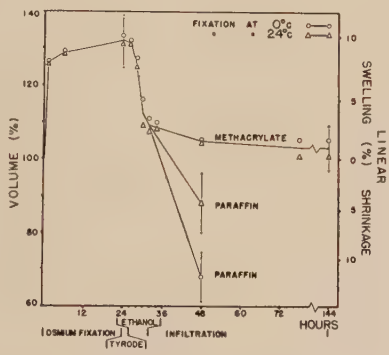
Measurements of the colloid osmotic pressure of some fluids were performed with a Hepp oncometer. With two samples of horse serum pressures of 321 and 319 mm H_2O respectively were obtained. These values are well within the range of normal serum pressures.

EXPERIMENTS AND RESULTS

Osmium fixation.—Fixation was performed with 1 per cent osmium tetroxide in balanced Tyrode's solution, buffered to pH 7.2, at temperatures of 0°C or 24°C. After 24 hours the specimens were rinsed in Tyrode's solution for 2 hours, then immersed for 2-hour-periods in 70, 95 and 100 per cent

ıol. Specimens to be embedded in paraffin were transferred to the ing agent. The remaining specimens were transferred to the metha- te and infiltrated with it for 2 hours. These specimens were then placed ethacrylate containing the catalyst. This procedure generally accords the well-established preparation techniques for electron microscopy are in use today ([30, 33], among others).





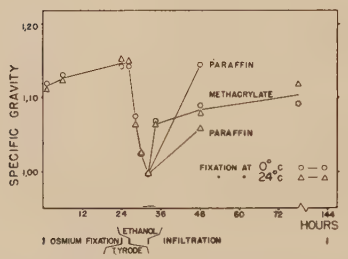
ig (*left*). Diagram showing the changes in weight, volume and specific gravity during fixation r r cent osmium tetroxide in Tyrode's solution at pH 7.2 for 24 hours.
ig (*right*). Diagram showing the changes in volume during fixation in 1 per cent osmium et ide at 0°C and 24°C followed by infiltration in methacrylate (0°C) or paraffin (58°C). The ur represent the means of four experiments. The mean ranges are indicated. Cf. Fig. 3.

ces of liver were fixed for 24 hours in the osmium tetroxide mixture n the changes were recorded at short intervals (Fig. 1). In this figure a ıa ed and rapid swelling is seen which reached a maximum of 30 per cent ft 4 hours. Half the final value was reached after only 15 minutes in the x ve. The course of the weight curve is rather similar to that of the volume u while the specific gravity rises slowly during fixation.

anges during the continued preparation are shown in Fig. 2. Here ou groups of liver specimens were investigated, corresponding to the v different fixation temperatures and two different embedding media. h experiment was repeated four times. The curves represent the mean of ıe four experiments. The ranges indicated on this graph represent the ıo s of the dispersion of individual experiments.

ter the initial rapid rise in volume there was a more gradual increase s ng as the specimens remained in the fixative. In the alcohols, on the th hand, there was a reduction of volume that almost restored it to the ri nal value. Infiltration with methacrylate caused but little further shrink-

age, even over a long period. In the four experiments, the final volume after methacrylate infiltration varied from shrinkage of 8 per cent to swelling of 20 per cent. Paraffin infiltration, however, gave rise to an entirely different picture; here the specimens shrank rapidly and considerably. This reaction was most conspicious with slices earlier treated at 0°C. The difference between specimens treated at different temperatures with regard to paraffin



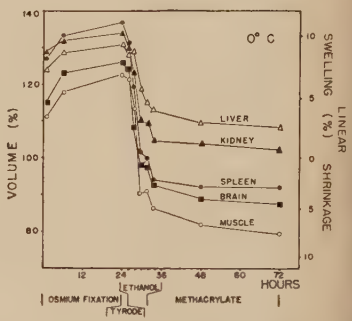


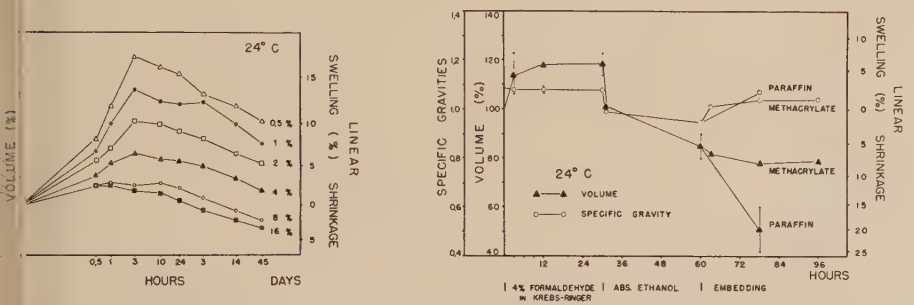
Fig. 3 (*left*). Diagram showing the changes in specific gravity during the same experiment as in Fig. 4 (*right*). Diagram showing the changes in volume of specimens from various organs. Fixation in osmium tetroxide at 0°C. Infiltration at the same temperature in methacrylate.

embedding was statistically significant. This, however, was not the case with the small differences noted during any other step. After paraffin infiltration the total shrinkage amounted to 25–45 per cent (0°C) and 9–14 per cent (24°C) respectively. The weight curves were closely consistent with the volume curves.

The specific gravities (Fig. 3) which rose slightly during the fixation, fell during dehydration and finally rose during infiltration. With the exception of the difference in specific gravities after paraffin infiltration, no significant differences were found with regard to temperatures.

To find out whether or not the results obtained with liver specimens were also representative of other tissues, specimens were taken from muscle, brain, spleen, kidney and liver, and treated as above. It was found (Fig. 4) that the volume changes of tissues other than liver exhibited similar characteristics, but some differences in intermediate and final values were noted. The extremes were liver with a swelling of 6–9 per cent and muscle with a shrinkage of 20–22 per cent. The weight curves, again, closely conformed with those for volumes. The specific gravities rose during fixation from 1.10 to 1.13 (average), fell during dehydration to 0.99, and rose in the methacrylate to a final value of 1.05.

maldehyde fixation.—The swelling observed during osmium fixation no way peculiar to this fixative. Fig. 5 shows the changes taking place eries of formaldehyde concentrations ranging from 0.5 to 16 per cent. pecimens remained in the fluid for 45 days at 24°C. In all concentrations ng occurred, the extent of which was inversely proportional to the ntration. Maximal swelling was reached after about 3 hours. After



left). Semilogarithmic diagram showing the changes in volume during fixation of liver ns in various concentrations of formaldehyde at 24°C.
right). Changes in volume and specific gravity of specimens during fixation in 4 per cent ehyde at 24°C, dehydration in ethanol and infiltration in methacrylate or paraffin. The represent the means of two experiments. The means of the individual experiments have rked.

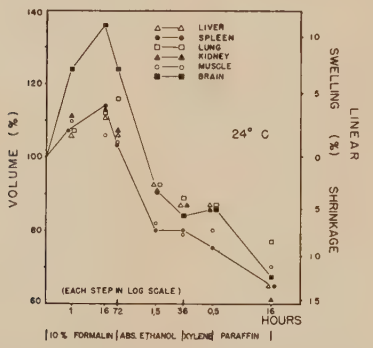
ncture a shrinkage took place, the rate of which was independent of ncentration of the fixation fluids.

purposes of comparison with osmium fixation, formalin-fixed speci- were observed through the same preparation procedures. Fig. 6 shows xation in 4 per cent formaldehyde caused swelling that reached a mum of 18 per cent after 12 hours. A considerable reduction of volume ed in ethanol (33 per cent) and paraffin (34 per cent). Methacrylate, on er hand, caused little shrinkage (8 per cent). The weights were affected ch the same way. The specific gravities of the specimens remained ally unchanged during fixation, dropped moderately in the alcoholic and finally rose close to the original value of 1.09 during embedding.

an additional experiment the behaviour of various tissues during in in 10 per cent formaldehyde was investigated. The tissues were t 24°C for 72 hours, dehydrated in ethanol for 36 hours, cleared in and infiltrated with paraffin. All tissues examined reacted in the same r, but certain differences were noted (Fig. 7). Brain tissues, for in- showed far greater swelling than any of the other tissues. Otherwise, nces between tissues were not significant.

Osmotic effects.—The results indicated that swelling in the fixatives was due not only to the fixing agents themselves, but in high degree to the aqueous media in which they were dissolved.

Specimens were suspended at 0°C in Tyrode's medium of various concentrations. Swelling occurred in all of them, including the strongly hypertonic ones (Fig. 8). Only with a tenfold concentration of Tyrode's medium



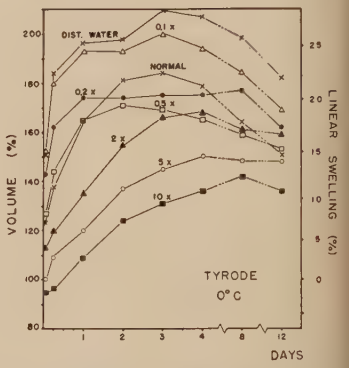


Fig. 7 (*left*). Volume of specimens from various organs during fixation in 10 per cent formaldehyde at 24°C, dehydration in absolute ethanol and infiltration in paraffin after clearing in xylene. The time for each treatment is indicated in logarithmic scale.
Fig. 8 (*right*). Swelling of liver specimens in hypo- and hypertonic Tyrode's medium at 0°C.

was slight initial shrinkage observed (first hour). The more the fluid was diluted, the greater was the swelling and the sooner it reached its maximum.

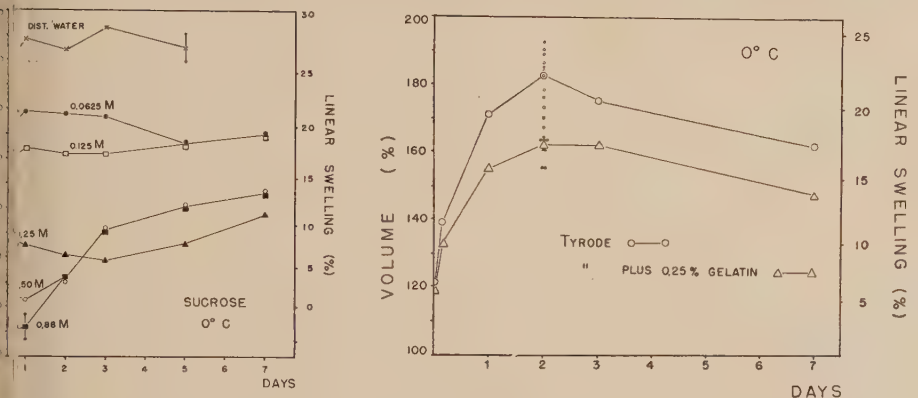
Similar experiments were carried out with saline and Krebs–Ringer bicarbonate or phosphate media, yielding results comparable to those obtained with Tyrode's medium. At a temperature of 24°C, the same swelling as that at the lower temperature was observed. It was found, however, that at 24°C a rapid decrease of volume occurred once maximal swelling had been attained after about 2 days. This regression may to some extent have been caused by autolytic and/or microbial effects.

Sucrose solutions are in general use as media for the isolation of cellular components. Although 0.25 *M* sucrose is regarded as isotonic, an 0.88 *M* solution is frequently employed. Fig. 9 shows that in sucrose media, too, swelling occurred that was inversely proportional to the concentration of the fluids. With 0.88 *M* sucrose, marked initial shrinkage was noted. All concentrations ultimately caused swelling.

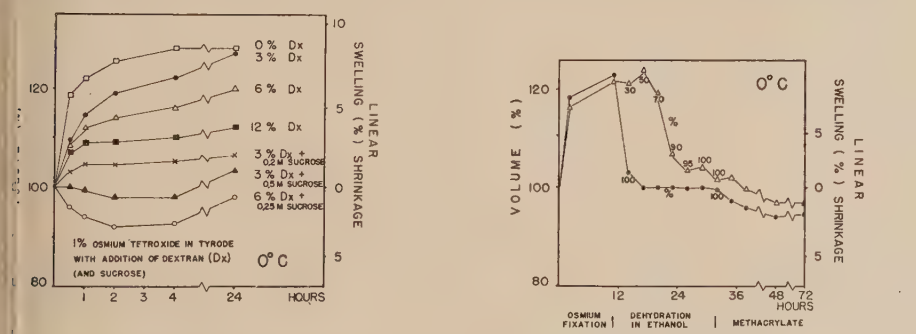
The addition of small amounts of gelatin to physiological fluids has been recommended [24] as a means of mitigating their deleterious effect on cells

g. 0 demonstrates that the addition of 0.25 per cent gelatin to Tyrode's
ɔm significantly reduced the swelling by about 20 per cent. The colloid
ɔtic pressure of this solution amounted to 54 mm H_2O.

Still more pronounced effect was noted with dextran, the well-known
a volume expander (Fig. 11). It was possible to use dextran in much



g. (*left*). Swelling of liver specimens in sucrose at different molar concentrations at 0°C.
g. (*right*). Effect of small amounts of gelatin on the swelling of tissue in Tyrode's solution.



g. (*left*). Diagram showing the effects on volume changes of the addition of different amounts
ntrations of dextran and sucrose to osmium tetroxide fixative.
g. (*right*). Diagram showing the shrinkage during dehydration after osmium fixation in
de ethanol and in series of rising ethanol concentrations.

gher concentrations than gelatin, without increasing the viscosity to an
ractical point. Nevertheless, merely an increase in dextran concentration
ld to counterbalance the swelling phenomenon, as demonstrated in Fig.
ombinations of dextran and sucrose, however, enabled the untoward
ng to be controlled.

Measurements of the colloid osmotic pressure of the dextran solutions

gave the following results: 3 per cent= 166 mm H_2O; 6 per cent= 310 mm H_2O; 12 per cent = 605 mm H_2O.

From Figs. 2, 4, 6 and 7 it is evident that considerable changes occur during dehydration. A closer study of these phenomena reveals that both the type of dehydrating agent and the sequence of concentrations play important roles. Thus it can be demonstrated that direct transfer of the fixed

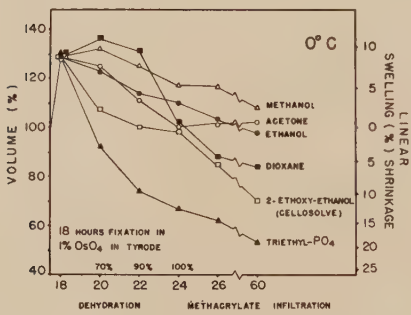


Fig. 13. Diagram showing the results obtained with various dehydrating agents after osmium fixation

material into absolute ethanol causes more abrupt shrinkage than does passage through rising concentrations of the same alcohol (Fig. 12).

Comparison of various dehydrating agents proposed in the literature [14, 15, 21, 25, 29] and miscible with methacrylate, suggested that with regard to volume changes only methanol gave better results than those obtained with ethanol (Fig. 13).

DISCUSSION

Osmium tetroxide has not yet been surpassed as a fixative for electron microscopy. Its advantages are well known to electron microscopists. In ordinary histology, however, its use has long been limited to the study of nerve and fat tissue. For this reason, our knowledge of its action is still very imperfect in spite of recent studies [2, 3, 11, 28, 30].

In the present investigation it was found that rapid swelling took place during fixation in osmium tetroxide. Increased weights and specific gravities of specimens were also recorded. This implies that swelling coincided not merely with infiltration of fluid, but also deposition of solids, viz. binding of osmium.

The swelling was caused by the aqueous media, even if hypertonic, but this effect was apparently counteracted by the stabilizing effect of the osmium tetroxide fixation, since the increase of volume was not so marked as that

normal Tyrode's medium. Furthermore, the termination of the rapid phase of swelling closely coincided with the cessation of osmium tetroxide uptake by the tissues, as revealed by chemical analysis [3].

During dehydration there was rapid and marked shrinkage in accordance with the effects of alcohols already described [6]. A further decrease in volume was effected by paraffin infiltration. On the other hand, methacrylate had no such an untoward effect. The end-result after osmium tetroxide fixation and methacrylate infiltration was a volume that did not deviate from the original value by more than a few per cent. Also, it is interesting to compare the differences in behaviour of e.g. liver and muscle with the uptake of osmium in these tissues, as examined by Bahr [3]. Liver has a high uptake, muscle a relatively low one.

Formaldehyde, for its part, has been extensively used in all types of histological techniques and our knowledge of its chemical action is relatively well founded [13]. We found that regardless of concentration, swelling occurred in 5–16 per cent formaldehyde solutions, although less pronounced with higher concentrations. Within the range of 4–10 per cent commonly employed, swelling for liver was less marked than with osmium tetroxide fixation. Shrinkage naturally took place in alcohol and paraffin, but infiltration with methacrylate caused but little reduction of volume. The entire course of the changes was very similar to that for osmium tetroxide fixation. The end-result after methacrylate infiltration for formaldehyde fixed material was, however, moderate shrinkage, whereas osmium-fixed specimens rather showed slight swelling.

Elkin and Shear [4] studied the comparative effects of serum colloids and of salts on the morphology of mouse tumor cells *in vitro*. They found that cell swelling took place in protein-free salt solutions. Increase of the salt content from below normal or hypotonic to above normal or hypertonic did not restrain the cell swelling: on the contrary, it promoted such swelling. A restraining effect on the swelling was noted when the solutions contained serum protein, but even then an increased salt concentration did not counteract swelling.

Comparable results were obtained in our investigations. Major swelling was found to take place in so-called physiological fluids of varying composition even if strongly hypertonic. These findings indicate—as has also been pointed out by Crawford and Barer [7]—that caution is required in applying the concept of isotony to the process of fixation. The intracellular concentration and composition of osmotically active substances may well differ from those of the extracellular fluid, osmotic equilibrium being maintained by

consumption of energy. This natural balance is definitely disturbed when the cell dies.

The presence of the fixatives, osmium tetroxide or formaldehyde, reduced the swelling that otherwise occurred in such media. This action cannot simply be explained on an osmotic basis, but is most certainly due to the chemical processes involved in fixation. The swelling during fixation could be diminished by substances of medium and high molecular weight, and in suitable concentrations they could entirely suppress this phenomenon.

It must be stressed that our experiments were performed with tissue blocks, though they were small in size. We are well aware that our results cannot simply be applied to the changes of individual components of the tissues examined. However, they coincide so closely with those obtained on individual cells [4, 7] that from these studies an idea can be gained of the volume changes during fixation. Three stages can be distinguished: (1) A brief initial period of slight shrinkage depending on the tonicity of the solution; (2) a period of re-expansion and swelling that may proceed to cell bursting (it may be attributed to a complex of factors such as intracellular salt concentrations, membrane permeability, Donnan equilibria and inability of the cells to continue active osmotic work); and (3) a period of secondary shrinkage, presumably due to a process of slow chemical alterations. In our experiments the stage of initial shrinkage was not observed in the fixation fluids, but only in very strongly hypertonic media. This stage is of short duration and is probably masked in the case of tissue blocks.

Volume changes also occurred in the further phases of preparation. These problems have also been studied by Seki [34, 35] who compared the action of numerous solvents during dehydration and embedding. Especially marked was the shrinkage during dehydration. This is difficult to eliminate since all dehydrating agents seem to have more or less the same effects. From our results it appears that some little benefit can be gained by using stepwise dehydration and exchanging ethanol for methanol. It has been shown [6] that methanol does not cause as much shrinkage as ethanol. With regard to temperature, it was found that this had but little influence on the volume changes during the treatment preparatory to electron microscopy. Hence, no evidence emerged in favor of fixation at low temperature. On the other hand, biochemically the lower temperature is obviously preferable. Morphological evidence supporting this view has also been presented [33].

Since our investigation was not limited to electron microscopic considerations, the sizes of the specimens and the durations of some stages may have exceeded those used by electron microscopists. However, our results clearly

the principal changes during the different preparation procedures. hat extent these changes will cause artefacts in the final preparations ot be stated, but it is reasonable to assume that such effects will be uced. Consideration of these matters is especially important in electron oscopy. It is well known that the present techniques for preparation of icroscopic specimens are not ideal, since a large proportion of speci-, although identically and carefully treated, have to be discarded, due e presence of artefacts.

me ways of avoiding untoward volume changes have been suggested. ner experiments are required, however, to evaluate the possible benefit ese measures. Such experiments would have to include volumetric ations on tissue blocks, microscopic examination of the changes in or small tissue blocks during treatment, and a careful electron micro- c analysis of the fine structure of the specimens.

SUMMARY

ie changes in volume, weight and specific gravity of tissue samples ng fixation in osmium tetroxide or formaldehyde and subsequent in- tion with methacrylate or paraffin were studied in more than 600 tissue mens. Substantial changes in volume and weight were found, and as e the weight changes closely parallelled the volume changes. The in- ce of the histological procedures on the specific gravity was not so ounced. During fixation in 1 per cent osmium tetroxide in Tyrode's ion at pH 7.2, a rapid increase of volume took place. Half of the final e was reached after only 15 minutes and the swelling was practically plete after 4 hours. After 24 hours it amounted to 30 per cent. Fixation rmaldehyde likewise caused swelling, which was inversely proportional e concentration (0.5–16 per cent). With 4 per cent formaldehyde in s–Ringer solution the swelling amounted to 18 per cent after 12 hours, half of this value was reached in about 30 minutes. Specimens left in ixation fluids for long periods displayed secondary shrinkage at a rate rently independent of the concentration of fixative. During dehydration anol, marked shrinkage occurred that averaged 23 per cent for osmium- and 33 per cent for formaldehyde-fixed material. Stepwise dehydration ed less abrupt volume changes, although the end-result differed but Among other dehydrating agents, miscible with methacrylate (methanol, one, 2-ethoxy-ethanol, dioxane, triethylphosphate), only methanol ared to give better results than ethanol. Infiltration with methacrylate

caused only slight shrinkage, around 7 per cent, regardless of the preceding fixative. During polymerization, however, methacrylate undergoes a 20 per cent reduction of its volume. As far as can be judged from planimetric observations, this shrinkage is to a great extent transferred to the specimens. Paraffin infiltration caused shrinkage of around 30 per cent. With osmium-fixed specimens the fixation temperature influenced the shrinkage during infiltration. Lower fixation temperature gave greater shrinkage. In other stages the temperature had little influence on volume changes. The swelling observed during fixation was found to be due, not to the fixing agents themselves, but to the aqueous media in which they were dissolved. When specimens were placed in saline, Tyrode's, Krebs–Ringer's and sucrose solutions, swelling took place in all of them, including the strongly hypertonic ones. The swelling was inversely proportional to the concentration and was less pronounced in sucrose than in the other medias. Addition of the fixatives, osmium tetroxide or formaldehyde, reduced the swelling to an extent that cannot be explained merely by osmotic action. The addition of macromolecules such as gelatin or dextran was found to reduce the swelling during fixation. The swelling phenomenon could be completely abolished if dextran and sucrose were added in suitable amounts to the osmium tetroxide solution. From the results obtained the following conclusions can be drawn. During conventional preparation procedures for histological specimens, major changes in volume, weight and specific gravity occur. Doubtless, these changes considerably affect the original geometry of the tissues constituents. It is possible, however, to reduce the volume alterations e.g. by addition of colloid-osmotically active substances.

We wish to express our gratitude to Mrs. M. Björkman, Miss G. Larsson and Miss G. Uppenkamp for skilful technical assistance. This work has been supported by a grant from the funds of Karolinska Institutet.

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A STUDY OF A CONTAMINANT IN AMPHIBIAN EGG EXTRACTS WHICH INTERFERES WITH THE DIPHENYLAMINE REACTION FOR DEOXYRIBONUCLEIC ACID[1]

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During the investigation of various aspects of nucleic acid metabolism in developing amphibian embryos, it became apparent that Schneider's hot trichloroacetic acid (TCA) extraction [8] of amphibian eggs and embryos yielded substances which interfered with the diphenylamine reaction for deoxyribonucleic acid (DNA). Although several investigators [2, 5, 6, 7] recognized the presence of interfering substances in various nucleic acid preparations, no simple method for eliminating these substances, or correcting for them, is in general use.

The purpose of this paper is to present evidence indicating the nature of the contaminant obtained from Schneider's hot TCA extraction of amphibian eggs and to describe methods for both eliminating and correcting for said contaminant.

Detection of Contaminant

Materials and methods.—The procedure outlined below was used until the presence of a contaminant which interfered with the diphenylamine reaction was detected. This procedure was then modified as described in detail in a later section of this paper.

Mature, unfertilized eggs from *Rana pipiens* were obtained by the pituitary injection technic [3] and manually stripped of jelly. The eggs were homogenized in the cold in cold 5 per cent TCA, centrifuged, and the precipitate was washed twice with cold 5 per cent TCA. In each case the supernatant fluid was discarded. The precipitate was then extracted thrice with hot ethanol ether (3:1) to remove the lipids. The precipitate, containing the nucleic acids and protein, was permitted to dry at room temperature. The nucleic acid derivatives were then extracted using Schneider's hot TCA procedure [8]. After centrifugation, a sample of the supernatant fluid was taken for the determination of DNA by the diphenylamine reaction [1].[3]

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[2] With technical assistance of John R. Newsome, Department of Physiology, Southern Illinois University, Carbondale, Ill., USA.

[3] The diphenylamine reagent was prepared by dissolving 1.0 g of diphenylamine in 100 ml of glacial acetic acid. To this solution, 2.75 ml of concentrated sulfuric acid were added.

ults.—There were two lines of evidence which suggested the presence ontaminant in our egg preparations:

In relatively dilute concentrations, the absorption peak of the diphenyl- e color complex was shifted from 600–605 mμ to the longer wavelengths.

A plot of concentration of egg extract vs. absorption at 600 mμ was not , the greatest deviation occurring at the more dilute concentrations.

hen a commercial preparation of DNA (Nutritional) was put through ntire extraction procedure and reacted with the diphenylamine, a color was developed which showed a peak between 600–605 mμ as ured on a Model DU Beckman spectrophotometer. TCA extracts from when reacted with the diphenylamine reagent, showed a different f spectrum. In this case, the peak was shifted to the 630–640 mμ range. e results are shown in Fig. 1.

hen dilutions of the egg extract were made and reacted with the diphenyl- e, a non-linear relationship existed when the optical density at 600 mμ was d against concentration of the egg extract. This is represented in Fig. 2.

Elimination of the Contaminant

our preliminary experiments it was found that when the TCA extract e eggs was put in the refrigerator for several hours, a heavy, flocculant pitate formed. When the extract was warmed to room temperature, or ly above, the precipitate went into solution again. Since this develop- suggested that the precipitate, which formed in the cold, may have the contaminant, the following experiment was performed:

th the precipitate in solution, samples were taken for reaction with enylamine. The remainder of the extract was then placed in the cold everal hours. The precipitate was centrifuged down and removed in old. Samples of the precipitate-free supernate were then used for the ion with diphenylamine. The results of this experiment are indicated mparing Figs. 2 and 3.

comparison of Fig. 2 and 3 indicates that when the precipitate is removed, linearity is obtained when the concentration of the egg extract is d against optical density at 600 mμ. It should be noted, however, that though the linearity is improved by the removal of the precipitate, it no means perfect. This suggested that the precipitation was incomplete hat some of the contaminant remained in solution.

was then decided to attempt a complete removal of the interfering ance(s). The criteria for the complete removal were selected to be the

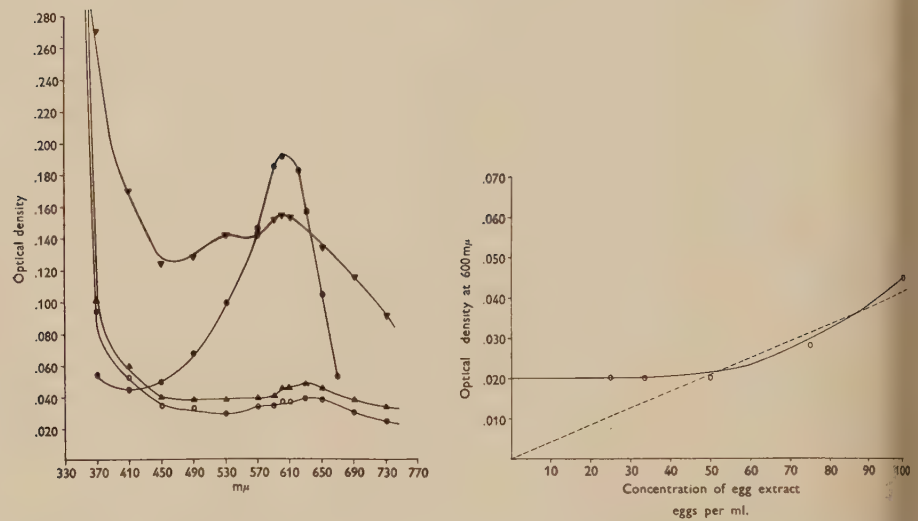


Fig. 1 (left). Spectra of diphenylamine color complexes. All samples had acid-soluble and lipid fractions removed as described in general procedure. 3 ml of 5 per cent TCA used for extraction at 90°C for 15 minutes. 2 ml of diphenylamine reagent added to 1 ml of extract and heated for 10 minutes in boiling water bath. ▼ 300 eggs/ml; ▲ 100 eggs/ml; ○ 50 eggs/ml; ● 130 μg commercial DNA/ml.

Fig. 2. (right). Diphenylamine readings vs. concentration of egg extract *before* removal of precipitate. Sample of several hundred eggs taken through entire general procedure and heated in 5 per cent TCA at 90°C for 15 minutes. Dilutions made of TCA extract to vary concentration. 2 ml diphenylamine reagent added to 1 ml of each dilution and color developed with 10 minute heating in boiling water bath.

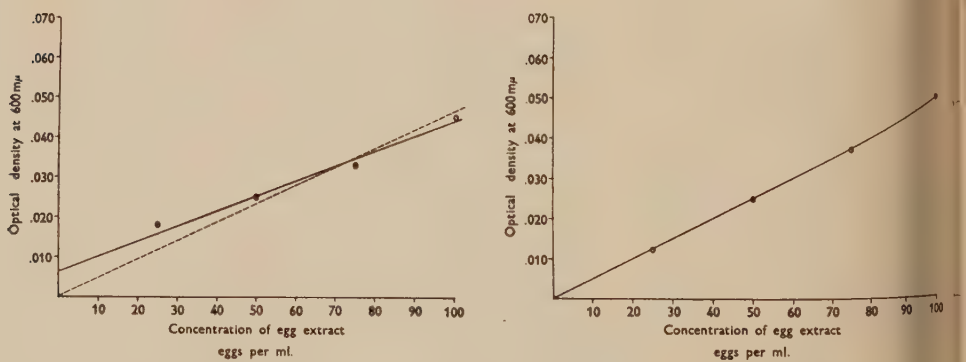


Fig. 3 (left). Diphenylamine readings vs. concentration of egg extract *after* removal of precipitate. Sample of several hundred eggs taken through entire procedure including 5 per cent TCA extraction at 90°C for 15 minutes. Sample then stored in refrigerator for several hours. Precipitate centrifuged off and supernate used for dilutions and diphenylamine reactions.

Fig. 4 (right). Diphenylamine readings vs. concentration of egg extract *after* Nuchar C unground charcoal column. Sample of several hundred eggs taken through entire procedure. 5 per cent TCA extract passed through charcoal column and dilutions made on filtrate.

ity obtained when the concentration of the egg extract was plotted st optical density at 600 mμ and the spectrum of the color complex of solutions of egg extract.

our experiments it was found that when the precipitate was isolated, ed several times in cold 95 per cent ethanol, dried, and dissolved in NaOH, it yielded a positive biuret reaction for protein. This result sted that it might be possible to separate the interfering molecules from oxyribose by use of a charcoal column. Consequently the TCA extract assed through a Nuchar-C unground charcoal column.[1] The column as washed several times with 5 per cent TCA and the washings pooled. ions of the washings were made and each reacted with the diphenylamine nt in order to test for linearity. Fig. 4 shows the results of such an experi-. The spectra of the 50 egg/ml samples showed a symmetrical peak at 10 mμ. These results indicate that the charcoal effectively removed the minating substance(s) from the TCA extract.

e next problem was to determine whether complete recovery of the oribose could be obtained from the charcoal column by washing with per cent TCA. This was accomplished as follows:

o 5 ml samples of commercial DNA (100 μg/ml) were made 5 per cent with spect to TCA and hydrolyzed at 90°C for 15 minutes. One sample was percolated rough the charcoal column and the other sample was kept as the control. The lumn was washed with 10 ml of 5 per cent TCA. The diphenylamine reaction was run out on both the control sample and the column wash. From this type of periment, it has been calculated that over 95 per cent of the deoxyribose can be covered from the column.

Correction for the Presence of the Contaminant

Our laboratory has been interested in determining accurately the DNA ount of less than 100 embryos. Although the charcoal column was highly effective in removing the contaminant, it was found unsatisfactory for our embryo work. The embryo samples were dilute to begin with and the added ume obtained from the column necessitated evaporation under vacuum concentrate the samples. This proved to be too time-consuming and obvious when a great number of samples were involved. Therefore, an attempt was made to prepare a correction curve for the presence of the contaminant. This was done in the following manner:

[1] The column was prepared by boiling the charcoal in 2 *N* HCl and storing under vacuum until use. The 0.5 cm × 8 cm column was neutralized before use by washing with distilled water.

The precipitated contaminant was "purified" by reprecipitation and washing with several volumes of ethanol and ethanol–ether. It was dissolved in 0.1 *N* NaOH. Aliquots were taken for biuret reactions and nitrogen determinations (Kjeldahl with direct Nesslerization). When these determinations were run on several dilutions of the contaminant, a straight-line relationship was obtained when biuret readings were plotted against micrograms of nitrogen. This is illustrated in Fig. 5.

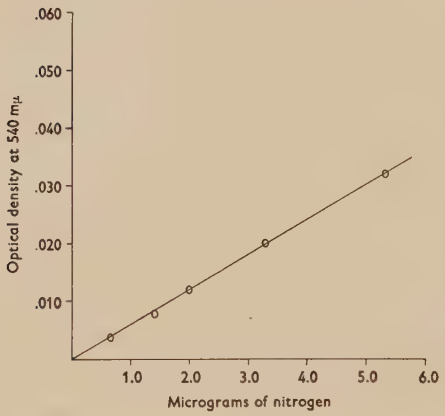


Fig. 5. Biuret readings vs. micrograms of nitrogen. "Purified" protein sample used for nitrogen determination using direct nesslerization. Then dilutions made of protein sample and biuret reaction carried out on each dilution.

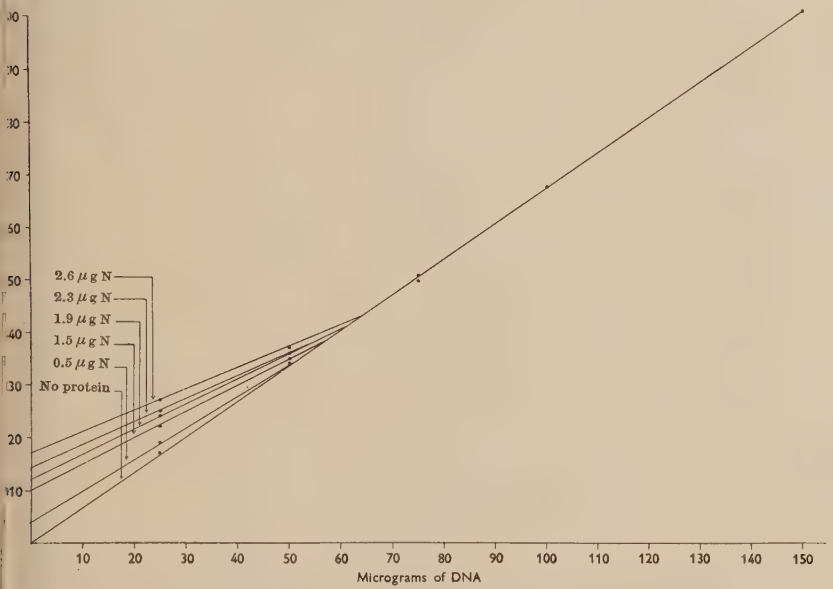
Next, a series of diphenylamine determinations was made on dilutions of commercial DNA which contained varying amounts of added protein. A biuret reading was taken on an aliquot of each protein sample and, from the graph depicted in Fig. 5, the protein nitrogen content of the sample was determined. A plot of the diphenylamine readings vs. micrograms of DNA with various amounts of protein yielded a series of lines as indicated in Fig. 6.

Fig. 6 indicates that when the protein nitrogen content is less than 0.52 μg there is little effect on the diphenylamine reaction. However, in a sample of 100 amphibian eggs which has been hydrolyzed in 3.0 ml of 5 per cent TCA there are approximately 2.3 micrograms of protein nitrogen in each ml of extract. As can be seen from Fig. 6, this represents a considerable error in the DNA determination.

This correction curve has been used in our laboratory as follows: From the 3.0 ml TCA extract of the embryos, 1.0 ml was reacted with diphenylamine and 1.0 ml with the biuret reagent. From Fig. 5 the micrograms of protein nitrogen corresponding to the biuret reading was determined. From Fig. 6, the curve which corresponds closest to the actual protein nitrogen content was used, along with the diphenylamine reading, to give the corrected amount of DNA in the sample.

Nature of the Contaminant

ent experiments have been directed toward an elucidation of the cal nature of this contaminant. This will be the subject of a future unication.



Correction curve for DNA in presence of contaminant. To commercial DNA varying amounts ified" protein sample of known nitrogen content were added. Diphenylamine reaction to all dilutions.

DISCUSSION

gelis *et al.* [4], in their paper on amphibian embryos, indicated that phenylamine color complex yielded a spectrum which differed from btained from commercial preparations of DNA. It was stated, however, his difference did not interfere with their DNA determinations. As hown previously, our work on amphibian embryos indicated that at concentrations a considerable error was introduced if the contaminant ot removed or corrected for. This is particularly important when ac- determinations of DNA are sought.

s not known whether the protein is derived from nucleoprotein or some other cellular protein. However, it is probable that the solubility cteristics of the protein (now denatured) change when treated with the CA. On standing in the cold, precipitation of most of the protein occurs.

It is yet to be determined if this protein is the interfering substance *per* or whether it is some other compound which is carried along through th extraction procedure or possibly liberated from a complex during extractio

As far as the protein itself is concerned, recent experiments indicate tha it is composed of only about eight different amino acids. These eight amin acids, when tested for their effects on the diphenylamine reaction, showe negligible effects. It is currently believed that after complete degradation t amino acids, the interfering nature of the material is lost.

SUMMARY

1. The presence has been found of a contaminant in amphibian eg extracts, obtained by Schneider's hot TCA procedure, which interferes wit the diphenylamine reaction for DNA.

2. Methods have been described for both eliminating this contaminant an correcting for it.

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DISTRIBUTION OF RADIOACTIVE PHOSPHORUS AMONG THE PROGENY OF A SINGLE LABELED BACTERIUM[1]

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With the advent of isotopes in biological research, ideas concerning the metabolic stability of the various molecular species in biological systems have undergone profound revision [22]. One came to view the amount of a biological substance in a living system to be the result of a "dynamic equilibrium" established by synthetic and degradative processes and to consider biological systems as being in a continual state of breakdown and renewal. To one concerned with a physical understanding of cell processes, it seemed important to find out whether such renewal processes really applied to all structures and substances within a given cell. Particularly was this true for materials concerned with genetic function and replication.

Deoxyribonucleic acid (DNA) appears to be at least one of the chemical constituents of a cell implicated in bearing genetic information. Suspicion that this is the case stems from its primarily nuclear locale, the constancy of amount of DNA in a particular class of nucleus under changing environmental conditions [5], the relation of DNA quantity per nucleus to ploidy [5, 19] and its actual presence in chromosomes. The hypothesis is strongly supported by the phenomenon of bacterial transformation [13, 30] and by the functional separation of DNA and protein in bacteriophage [12]. Experiments concerned with the renewal of DNA have led to some conflicting reports in the literature [1, 23, 6, 2, 20, 24, 7]. At least with normal precursors, there appears to be general agreement that there is no synthesis or degradation occurring in resting cells [21, 4, 9]. As a corollary, DNA synthesis and renewal, if there is any, seems to occur only in cells which are stimulated to divide. In the case of bacteria [11] and of cultures of animal cells [10] in the log-phase of growth, strong evidence has been presented that no renewal of pre-formed DNA occurs. Whether this represents a special situa-

[1] Research in part carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

[2] Present address is the latter. Part of the work was performed during temporary residence at Boyce-Thompson Institute, 1086 North Broadway, Yonkers, New York.

tion where, due to the generally rapid growth conditions, potential degradative processes are necessarily minimized, or whether, in fact, there are no degradative processes for pre-formed DNA in the normal course of a cell's metabolism has not been established.

The lack of renewal of DNA indicates nothing about the distribution of atoms in parental DNA between the old and newly synthesized DNA. Investigation of this question for bacteriophage by Levinthal [16] revealed a large piece of DNA which maintained its integrity during replication. The results were interpreted as evidence in favor of a Watson-Crick [27] type of replication. The relationship between the directly demonstrable genetic function of DNA in the cases of the bacterial transforming principle and bacteriophage and the presence of DNA as only one of the components of the apparently functional genetic unit of higher animal and plant cells, the chromosome, is not at present clear. Current evidence from the bacterium *Escherichia coli* K-12 suggests that genetic information may be linearly ordered in a few [28] or possibly only one structure per nucleus [29]. Whether this structure can be thought of as one long strand of double-helical DNA or whether the integrity of linearly arranged genetic information depends on some more complex structure or process are questions wanting answers.

It would seem useful to study the distribution by radioautographic means of radioactive phosphorus among the progeny of a single labeled bacterium growing on non-active medium. One might hope to find evidence for or rule out subdivision of the cell DNA into a relatively small number of large organizational units whose integrity with respect to radioactive phosphorus would be maintained through cell division. One would not expect the other major phosphorus-containing components of the cell to be organized into large units. Consequently, it might be hoped that, following sufficient multiplication, dilution of these non-DNA components among the progeny would permit the observation of those cells containing large DNA subunits as particular "hot" cells.

The organism chosen for this work was *Micrococcus cryophilus* [18], a coccus which has the convenient property of separating cleanly into individuals at division rather than clumping or forming chains. Furthermore, from cytological observations, the spherical cells obtained following divisional separation would seem to have at most two nuclear bodies [3, 14], a situation which seems simpler than is true for most rod-shaped bacteria. After preliminary experiments with washed radioactive cells, regrown in non-active medium and examined after growth for "hot" cells, it became apparent that the most satisfactory way of handling the problem of asynchronous

h and even cell death as contributing factors to non-random P^{32} distri-
n among the progeny was to turn to micromanipulative isolation of all
rogeny of a single labeled bacterium.

MATERIALS AND METHODS

organism used was a strain of *Micrococcus cryophilus* kindly supplied by Dr.
d D. Delamater. For much of the work a clonal variant was isolated by micro-
ulation, which, by virtue of its ability to stick to glass microneedles, proved
larly useful in manipulation. The cells were grown at room temperature (about
in 1 per cent (w/v) Bacto-peptone solutions to which was added P^{32}* as ortho-
ate to the desired specific activity (from 7 to 80 μc P^{32} per μg of P^{31} in different
ments).

chemical extraction procedures on slides, aliquots from the radioactive cultures
ashed two times by sedimentation and resuspended in non-radioactive nutrient
Smears on microscope slides were made immediately and air-dried. The slides
ipped into a solution of 1 per cent parlodion in 50:50 ether–alcohol and dried.
rocedure provided a thin film of parlodion which, by the results of the extrac-
rocedure and by microscopic observation of the cells, seemed to keep the cells
ce during the extractions without measurable interference with the extraction.
and alcohol-soluble phosphorus was extracted by immersion of slides in 3:1
alcohol and glacial acetic acid for 20 minutes, followed by two-minute washes
ries of ethyl alcohol–water solutions, finishing with water. Such a fixing proce-
removed about 15 per cent of the cells' phosphorus. The same result could be
ed using 5 per cent cold trichloroacetic acid in place of the alcohol–glacial acetic
ep. Hydrochloric acid extractions were carried out in 1 *N* acid at 60°C. Ribonu-
(RNase) (Worthington) solutions contained 0.2 mg of enzyme per ml at pH 6.0.
ribonuclease (DNase) (Worthington) solutions contained 0.1 mg of enzyme
l and 0.005 *M* $MgSO_4$ at pH 6.0. Control solutions were without the enzymes.
he hydrolyses were performed at 37°C.

micromanipulation, small drops of a radioactive culture containing log-phase
hich had undergone at least one-hundred-fold growth in the radioactive medium
eposited on thin layers of non-radioactive nutrient agar (1 per cent Bacto-Agar,
cent nutrient broth) on coverslips, which were then placed in moist chambers.
ons of individual cells for controls and for progeny line studies were made
glass microneedle and washing of radioactive medium was accomplished by
l transfers in position of the initially isolated cells on a second block of non-
ctive agar. Spacings between individual cells were always kept large enough
imize cross-fire in subsequent radioautography. After manipulation, the agar
ied down, the coverslip parlodion-coated and fixed. Radioautography of such
lips was performed with the stripping-film technique [8] (Kodak Ltd., London,
nd). The developed emulsions were mounted for microscopic grain-counting in
er cent glycerin solution in water which resulted in an emulsion layer expanded

* P^{32} was obtained from Oak Ridge, Tennessee and from Harwell, England, on allocation from
omic Energy Commission.

to about 10 microns in thickness. Grain-counting was performed at 2000 × magnification under oil with illumination for minimum depth of focus (Kohler). A Whipple eyepiece reticle was used and grains were counted in planes 2 microns and 6 microns above the plane of focus of the bacterium.

EXPERIMENTAL

Phosphorus components of Micrococcus cryophilus.—The phosphorus components of the cells were determined by counting the radioactivity with an end-window counter of smears on slides of randomly labeled cells before and after extraction procedures. Study of the time course of phosphorus removal during such extractions yielded a consistent picture of the phosphorus components of the cells. Fig. 1 shows the removal of phosphorus from fixed smears during HCl hydrolysis. Each point represents results from a single slide. At least two components exist, the second accounting for about 20 per cent of the phosphorus of previously fixed cells. After 10 minutes hydrolysis, the cells are Feulgen positive. (The Feulgen reaction was kindly run by Dr. Montrose Moses.) The expectation is that the fast component is attributable to ribosenucleic acid (RNA) phosphorus while the slow component represents the more slowly hydrolyzing DNA phosphorus. Confirma-

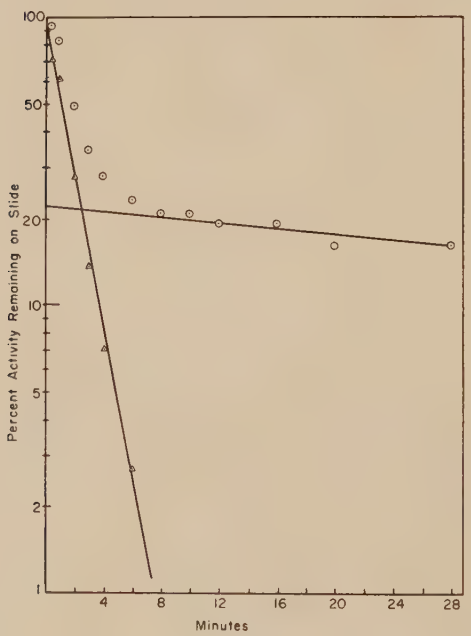


Fig. 1.

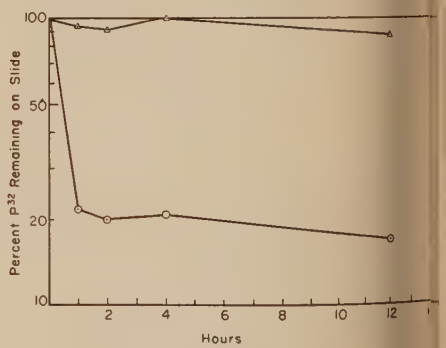


Fig. 2.

Fig. 1. Release of P^{32} during hydrolysis of labeled *Micrococcus cryophilus* in HCl at 60°C. O, per cent P^{32} remaining on an individual slide; △, fast component, obtained by subtracting visually estimated slow component from data points.

Fig. 2. Extraction of P^{32} from *Micrococcus cryophilus* with ribonuclease. △, controls; O, enzyme.

f this was achieved by enzyme hydrolyses. RNase, acting on fixed cells, a a resistant component of the same size as HCl hydrolysis. (Fig. 2). o 70 per cent of the phosphorus remaining after RNase is removable Nase (Fig. 3). The nature of the fraction resistant to DNase was not ined though presumably it could contain phosphorus due to DNase- si nt DNA, alcohol-insoluble lipid and/or protein phosphorus. Thus, a

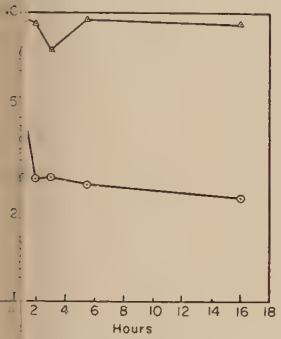


Fig. 3.

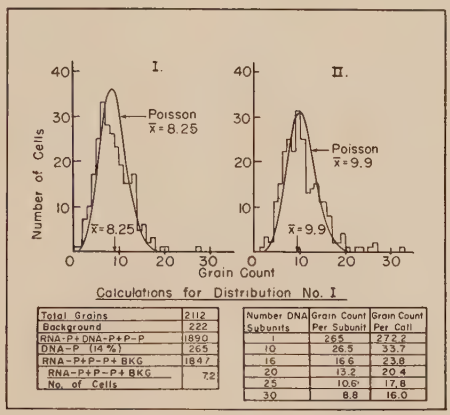


Fig. 4.

Extraction of P^{32} from *Micrococcus cryophilus* with deoxyribonuclease. △, controls; ○, y.

P^{32} distribution among 8th generation progeny of a single labeled cell of *Micrococcus us.* $\bar{x}$, average grain count per cell. See text for discussion.

imum of 14 per cent of the phosphorus in fixed cells can be attributed to N The phosphorus fractions were determined in the above manner rather a by bulk chemical procedures in order that they might represent more s the status of cells actually used for radioautography in this as well i future experiments.

Progeny-line phosphorus distribution.—It is desirable to have the simplest s le starting situation for progeny-line study. Therefore, a droplet of d ctive cells was deposited on agar in a moist chamber and, from this, h cal cells which had just completed divisional separation after deposition a agar were selected. Progeny-line isolations were then made through e sired number of generations. The daughters of a single cell do not u y stay in perfect synchrony under these conditions of growth. Generally, o several starting cells in one moist chamber, those cell lines which stayed s t to being synchronous were selected for further manipulation and t aphy. Although studies of clones from the third to eighth generations

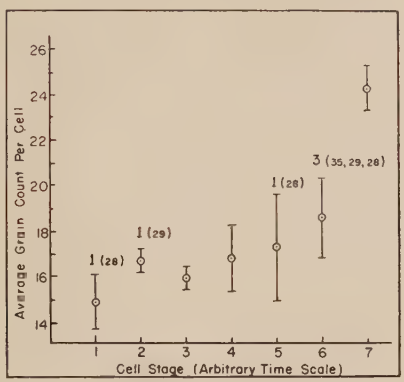
were made, this report will primarily be concerned with the eighth generation results, since information embodied in this generation is inclusive of that in earlier generation data. Fig. 4 shows the grain count frequency distribution for eighth generation cells for two different clones. Because of asynchrony in the second clone only the data of 240 cells is presented, the remaining 16 having proceeded to the ninth generation. There were, however, no particularly hot cells among these advanced cells. For comparison, Poisson distributions about the same average grain count as that shown by the data are plotted.

Since the main interest in these experiments was the question of the organization and integrity of the original cell's DNA, calculations of interest are included in the figure for the data of the first clone. These calculations present the expected average grain count of cells containing labeled DNA, assuming a model which considers the original cell's DNA to be subdivided into independently segregating but metabolically stable subunits, n in number and each containing 1 nth the original DNA, with the subunits maintaining their integrity with respect to phosphorus on replication. Comparison with the data would make it seem unlikely that any model based on $n < 25$ would fit.

The data deviates considerably from a Poisson distribution. It cannot be ruled out that some of this deviation is due to non-random physical processes in autograph registry of the actual P^{32} distribution in the cells. However, there appear to be at least two biological processes contributing to the deviation. In the course of manipulation, it is possible to classify cells as to their stage in the division cycle by arbitrary visual criteria. Since, as stated previously, there is some asynchrony in every cell line, a correlation of grain count can be made with cell stage recorded at the finish of a clonal manipulation. A typical plot of such averaged grain counts for cells of a particular stage against an arbitrary scale corresponding to increasing advancement in division cycle is shown in Fig. 5 for a seventh generation experiment. Although the form of the curve is not considered significant, in every case examined thus far, the average grain count of the fastest growing cells was higher than that of the slowest growing cells. Since about 80 per cent of the grain count in these distributions is the result of RNA phosphorus, it seems not unlikely that one is observing a relationship between the rate of growth of individual cells and divisional asymmetry in RNA distribution between daughters.

That this asymmetry is small at any one division is shown by control experiments designed to test the autograph registry. In such an experiment,

droplets of randomly labeled cells deposited on non-radioactive nu- agar, cells which were just about to undergo divisional separation were ed, allowed to divide, the daughters separated and radioautographed. h experiment about 32 such daughter pairs were used. For comparison expectations from random splitting of the phosphorus between daugh- calculations were made of the χ^2 value for each pair of cells. These



g. Comparison of cellular P^{32} content with rate of wth. See text for discussion.

dual values were summed for all the pairs in an experiment. For w experiments, the chance of obtaining from random splitting a χ^2 value r than that from the experiment was never less than 10 per cent; that , we could not statistically distinguish the splitting as differing from random ing. Consequently, any inequality in phosphorus-mass distribution at ny one division must be small. The correlation of P^{32} content with growth over several divisions must be the result of the accumulated effects of n asymmetries in phosphorus distribution at any one division, com- ded over several divisions.

Examination of the hottest cells in the progeny grain-count distributions ls that usually not all of these cells are among the fastest growing. In one frequently finds some of these hotter cells amongst the slowest ers. The numbers followed by parenthetical figures above the data is in Fig. 5 indicate the number and actual grain count of the six hottest ll in this seventh generation clone. In this case, two of the hot cells are g the slower growing cells. Such observations, though they may be ical accidents, especially in view of the uncertainty as to the perfection tograph registry, lead one to suspect a second biological process pos- associable with DNA partition at division. Preliminary experiments on autographed after HCl hydrolysis may support this interpretation.

The results indicate an asymmetry in DNA P^{32} distribution in some of the division pairs of clones allowed to grow for five generations from a single radioactive cell. The details of these experiments will be the subject of a future communication.

DISCUSSION

Ideally, one would like to know that the radioautograph grain number accurately reflects the amount of isotope in the individual bacteria. Since there are many potential hazards in the autograph registry which are difficult to assess on theoretical grounds (e.g. non-random distribution of sensitive halide in the emulsion; variation in grain density with electron energy together with the distributed nature of isotope β-energies), one must rely on suitable control experiments. In the region of grain-density used in the work, experiments were performed which showed that the autograph grain count appeared to be satisfactorily a linear function of decay-corrected exposure, a result in agreement with findings of others [26]. However, for the type of result reported here, one would like to have a set of radioactive point sources each of which was known independently to have the same amount of phosphorus with the same specific activity. Two methods of selecting such a set of sources from randomly labeled bacteria were tried. Isolation of cells of *M. cryophilus*, which by visual criteria were at a particular stage of the division cycle, did not consistently yield Poisson grain-count distributions. Likewise, autography of spores of *Bacillus subtilis*, where it might be hoped that one might obtain the ideal set of sources on the basis of their biological characteristics and on the basis of exceptional uniformity to inactivation by heat and X-rays (Morowitz, unpublished data), did not yield grain-count distributions with satisfactory Poisson fits. The failure in both of these cases could be biological rather than physical; that is, our selection procedure may not provide the ideal set of sources because the cell populations selected may not, in fact, be homogeneous in phosphorus content. The only type of control which proved statistically satisfactory as evidence for reliable autograph registry was that performed with paired daughters as described above. Though such results give one considerable confidence in the method, they still leave in doubt the magnitude of registry errors contributing spread to the cell grain-count distributions as reported. It must be noted, however, that the conclusions drawn from the data are independent of such potential errors except where mentioned in presenting the data.

The main conclusion to be drawn from this work centers around the cal-

ul ons in Fig. 4. Certainly one must rule out the organization of total
ac rial DNA into a small number of equivalent structures which would
g gate independently during the cell divisions and which would maintain
ne integrity with respect to phosphorus. Thus, structural integrity through
v al division cycles of the total DNA of a bacterial nucleus or even of
os le chromosome-like structures in the nucleus seems unlikely for this
g ism.

l s possible that the size of units which might maintain integrity is con-
d ably smaller than that represented by $\frac{1}{25}$ of the total bacterial DNA.
in the DNA content of *Micrococcus cryophilus* is about 10^{-14} gm/cell,
c he bacterial DNA is about twice the amount of DNA in T2 *Escherichia*
b acteriophage. Levinthal's data indicates that the largest piece to be
ra ained intact on replication is only 20 per cent of the DNA of the phage.
i lso conceivable that there may be considerable heterogeneity in the size
f NA subunits and their fates, such that only the larger and stable pieces
ou show up in an eighth generation study as performed. The hottest cells
i e tails of the distribution might represent cells containing such pieces.
Ho ever, one can certainly rule out the existence of any one piece containing
no than 10 per cent of the initial cell's DNA P^{32}. It may be that a cell's
N is organized into a relatively small number of segregatable packets,
th as large DNA pieces, or, as smaller DNA parts of larger superstructures
ch mosomes), but that some process such as cross-over occurs frequently
no h in the course of replication that only parts of the original units retain
ne integrity through repeated replications. The preliminary acid hydrolysis
ex iments mentioned above are suggestive of this. The results obtained
vo aut [17] using meristematic cells in plant tissue with C^{14} thymidine as
sp cific DNA label might not be inconsistent with this possibility.

I the calculation of Fig. 4, it has been assumed that there has been no
os or redistribution of the P^{32} among the phosphorus components of the
ce These questions have not been thoroughly investigated for this organism
th gh in other bacteria such phenomena are not observed [11]. Since the
ex iments were designed to test the question of the presence or absence
f atively large stable organizational units for the cell DNA which might
ma tain their integrity with respect to phosphorus during several bacterial
di ons, any loss from the cell of DNA P^{32} would only confirm the result as
re nted for the total DNA or at best force the acceptance of a dichotomy
f es for cellular DNA. A redistribution of P^{32} from other components into
N should not affect the calculations for the original DNA P^{32}. Loss of
^{32} om other fractions would imply that the calculated minimum number

of stable subunits would have to be greater to fit the data. Redistribution of fix-soluble phosphorus into non-DNA fix-insoluble material could at best lower this minimum estimate of number of subunits by about 15 per cent.

SUMMARY

The distribution of radioactive phosphorus among the progeny of a single labeled cell of the bacterium *Micrococcus cryophilus* has been studied through the eighth generation progeny by using micromanipulative and radioautograph techniques. The DNA of this bacterium cannot be organized into twenty-five or less equivalent stable subunits whose phosphorus integrity is maintained on division. There cannot be one such subunit with more than 10 percent of the cell DNA. The rate of growth of individual bacteria seems to be correlated with a small asymmetry in phosphorus (possibly RNA phosphorus) distribution at division.

I wish to thank A. David Wolfe who performed much of the chemical extraction work and in general helped to clarify many issues with his discussions; Anne-Lise Dahlsrud for her capable technical assistance; Donald Fluke and Phelps Crump of Brookhaven National Laboratory, Vaughn T. Bowen, formerly of Brookhaven National Laboratory, and Harold Morowitz and Walter Guild of the Yale Biophysics Department, for interest and discussion.

My most heartfelt thanks must go to Ernest C. Pollard, chairman of the Biophysics Department of Yale University, for his patient encouragement and guidance throughout the course of this work.

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ON THE EFFECT OF PORPHYREXID AND PORPHYRINDIN ON THE FERTILIZATION OF THE SEA URCHIN EGG

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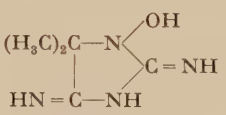
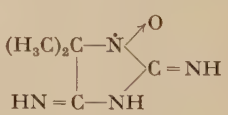
It has been demonstrated that periodate (10^{-6}–5.10^{-5}) brings about a facilitation of the fertilization of the sea urchin egg [8]. This is due in the first place to a change of the egg surface, as pretreatment with the reagent sufficed to bring about the effect. Moreover, it could be demonstrated that the rigidity of the egg surface decreases strongly upon pretreatment with periodate. A treatment of the eggs with somewhat higher concentrations of the reagent causes a membrane elevation followed by nuclear activation [9]. The same result was obtained upon prolonged treatment [8]. It seemed important to compare the effect of periodate with that of other oxidizing agents in order to attain an interpretation of the mode of action of periodate. Therefore Runnström and Hagström [16] tested a certain number of other oxidizing agents. Their conclusion was that periodate seemed to be unique in its action. The present writer continued, however, to look around for oxidizing agents which could be tested with respect to their action on the fertilization of the sea urchin egg. By courtesy of Dr. Hans Bielig (Heidelberg) I got the possibility of testing the effect of porphyrexid and porphyrindin which were introduced by Kuhn and Franke [10] as oxido-reduction indicators of unusually high normal potential, viz. 0.725 and 0.565 V at pH 7.0.

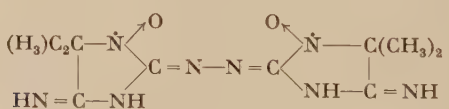
Below the formulae are given of porphyrexin (I), porphyrexid (II) and porphyrindin (III), cf. [10], since this will help in discussing the results obtained. Porphyrexin is the precursor of porphyrexid in the chemical synthesis of this indicator; porphyrexin has also been tested with respect to its action on the eggs.



I ($M = 142$)



II ($M = 141$)



III ($M = 280$)

MATERIAL AND METHODS

T gametes of *Paracentrotus lividus*, *Psammechinus microtuberculatus* and *P. mi-* served as material in this research. The two first-mentioned species were ec uring a stay at the Stazione Zoologica of Naples in the spring 1956, whereas e ter species was used in work carried out in the summer 1956 at the Zoo- Station, Kristineberg, on the west coast of Sweden. The eggs and sperm e btained by removing the gonads. In the costumary way the eggs were filtered r h bolting silk, whereas the outflowing sperm from the testes were collected i pipette and brought into a narrow test tube which was stored in a refrigerator. e lution of the concentrated sperm was made in such way that the final concen- at was 2–3 $\times 10^6$ spermatozoa per ml egg suspension. In the tables n means the r of eggs counted.

T solutions of the indicators were always made up in sea water. Washing with e a water subsequent to exposure to the indicators meant a dilution of these actor of 10^2–10^3.

RESULTS

Insemination of Eggs in the Presence of the Indicators

I these experiments the eggs were immersed in a number of different ntrations of porphyrexid or porphyrindin. After different intervals of sperm was added and the eggs were examined in the microscope. the headings 1 A and 1 B such experiments are described in detail.

In one experiment with *Paracentrotus lividus* the test solutions had the follow- lar concentrations of porphyrexid:

a	*b*	*c*	*d*
0	1.4×10^{-4}	7×10^{-5}	3.5×10^{-5}
e	*f*	*g*	*h*
1.75×10^{-5}	1.05×10^{-5}	5.6×10^{-6}	1.4×10^{-6}

exposure of the eggs to the test solutions for an hour the eggs were inseminated. , the control in sea water, 100 per cent of the eggs were fertilized with a well- ed membrane. Conversely, no fertilization occurred in *b*–*g*. Only in the test solu- did fertilization again approach 100 per cent. However, the type of membrane ion is highly atypical. The membrane is only slightly separated from the egg e. In this latter a concavity is formed above which the refractile membrane ole, see Figs. 1 and 2. Very often two concavities are formed instead of one. oncavity or concavities appear 2–3 minutes after insemination and get more d in the following minutes. In the space between the concave part of the egg e and the membrane one often observed connecting strands or rods of the kind usly described [13].

The situation in *a*, *g* and *h* was the following 170 minutes after insemination:

	Divided eggs per cent	Non-divided eggs per cent	*n*
a	100	0	> 200
g	0	100	> 200
h	74	26	191

The qualitative difference between *a* and *h* was considerable. In the former regular 8-cell stages, in the latter irregular 8-cell stages with a rather tight membrane were observed. The non-divided eggs of *h* were practically all fertilized.

The eggs of *a* developed into normal plutei. In *h* the divided eggs also developed into plutei of rather normal type. Some few animalized larvae were observed among the plutei.

1 B. On the same material of gametes an experiment was carried out which forms a parallel to the previous one. In the present experiment, however, the eggs were inseminated *one minute* after the transfer to the test solution.

In the control 100 per cent fertilization was noted and the membrane was well elevated. In *b–e* no membrane formation occurred. In *f* fertilization occurred in a minority of the eggs. In *g* the conditions were very similar to those prevailing in *h* of the experiment reported in 1 A. Thus the membrane was closely adhering. Concavities were observed above which the membrane is particularly visible etc. In *h* close to 100 per cent of the eggs had a fertilization membrane of the same kind as that of the control *a*.

The situation was the following 105 minutes after insemination:

	Divided eggs per cent	Non-divided eggs per cent	*n*
a	100*	—	> 200
f	8**	92	186
g	48***	52	213
h	100*	—	> 200

* Only 4-cell stages.

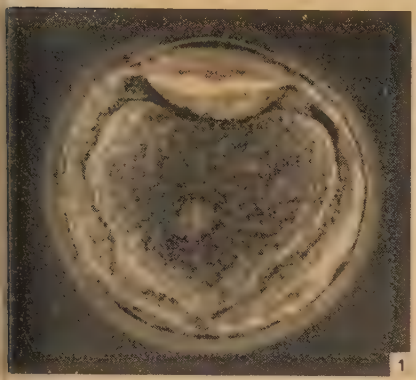
** 2-cell stages with tight membranes and blastomeres pressed against each other. Some few 4-cell stages.

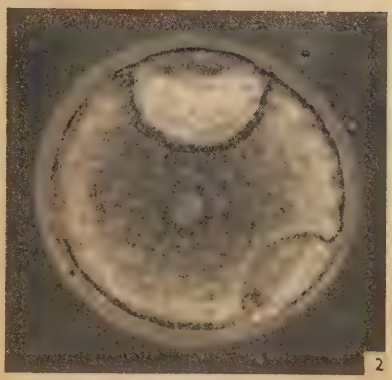
*** Mostly 4-cell stages.

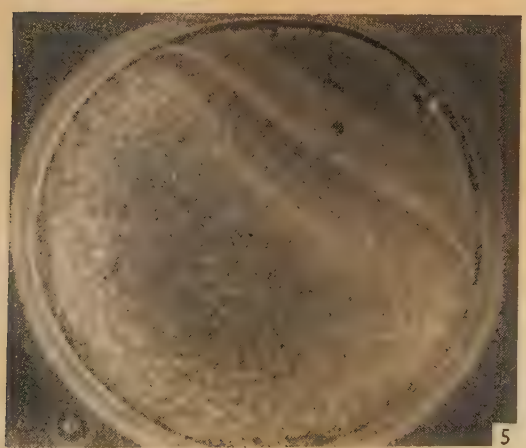
In the blastula stage the epithelial wall was thicker in *f–h* than in *a*. Nevertheless plutei developed in *g* and *h*.

Figs. 1–2. Eggs of *Paracentrotus lividus* fertilized in the presence of 1.4×10^{-6} *M* porphyrexin. Formation of one or two concavities. Phase contrast.

Figs. 3–5. Eggs of *Paracentrotus lividus* pretreated for 4 hours with 3.6×10^{-5} *M* porphyrindin, washed in sea water and then inseminated; 60–70 minutes after fertilization. Phase contrast.







These experiments showed that the inhibition of the fertilization is complet down to the concentration 10^{-5} *M*, irrespective of whether the eggs have bee treated with the reagent for 1 or 60 minutes before insemination. Only belo 10^{-5} *M* were differences found which indicate that there is a time facto involved in the action of the reagent.

Insemination of Eggs Pretreated with the Indicators

In a number of experiments the eggs were pretreated for varying interval of time and were thereafter washed in normal sea water and inseminated i this medium.

2A. In one experiment with *Paracentrotus lividus* the times of transfer were th following:

I	II	III	IV	V
10 min.	30 min.	2 hours	4 hours	25 hours

The following porphyrindin concentrations were used:

a	*b*	*c*	*d*	*e*
0	3.6×10^{-5}	2.2×10^{-5}	1.0×10^{-5}	5.2×10^{-6} *M*

Thus the experiment was subidvided after transfer to normal sea water into 25 dif ferent tests: I*a–e*, II*a–e*, etc.

The eggs were inseminated 5–15 minutes after transfer to normal sea water. I I–IV fertilization occurred in all eggs irrespective of the pretreatment with the indica tor but the membranes were low or sometimes irregular in the pretreated eggs particularly if the two higher indicator concentrations (*b*, *c*) had been used.

There was definitely an increased effect on the membrane formation with prolonga tion of the pretreatment but it was difficult to bring this on quantitative terms.

In I*b* the *membranes* were low and irregular; the eggs became angular after fertiliza tion.

In II*b* the membranes were more adhering and one or two concavities appeare in the egg surface, cf. Fig. 1. In III*b* and IV*b* the number of eggs with concavities increased further. Sometimes strands were noted which extended between the egg surface and the membrane. These correspond to deformed elongated cortical granules cf. Figs. 3–5.

Along with the strands rods or angular plates may be seen in the concavities. The structures in question may sometimes have curved or spiral shape. They correspond to transformed cortical granules (cf. [13], p. 58).

The abnormal features of the membrane formation decreased in eggs which had been pretreated with the lower concentration of the indicator. In I*e*–IV*e* finally the membrane resembled that of the control. It was slightly less elevated.

In I*b* and II*b* all eggs underwent cleavage.

In II*b* and IV*b* a number of eggs remained undivided despite the formation of a membrane. In these eggs a monaster was formed.

The eggs were distributed in the following way in IV*b* and IV*c*, 80–90 minutes after insemination:

	Divided eggs	Monaster eggs	Non fertilized eggs	*n*
IV *b*	56 per cent	41 per cent	3 per cent	288
IV *c*	88 „ „	12 „ „	—	210

Monster eggs were very rare in IV*d*.

The series V deviated from the series I–IV by the fact that no membranes were formed in the control, V*a*. This was due to a disintegration of the vitelline membrane which was often observed in eggs kept for 20–25 hours at room temperature.

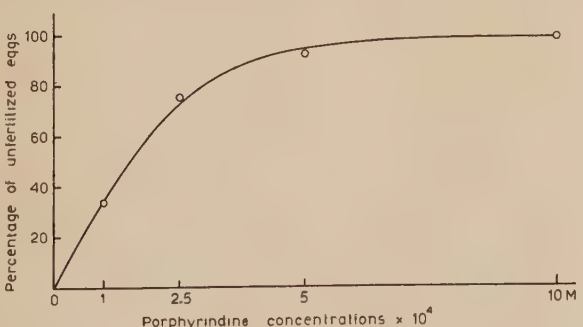
In V*b*, on the other hand, a membrane appeared in 100 per cent of the eggs. The membrane was of the same type as described for II*b*–IV*b*. The membrane is low but may exhibit local elevations where the membrane is more refractile and where atypical fusion of the cortical granules, strands or rods, were visible.

The first division commenced in more than 50 per cent of the eggs. The tightness of the membrane allowed no elongation of the egg at telophase and the furrow formation must have regressed, because a later count gave only 13 per cent of cleaved eggs (V*b*). The per cent cleavage was approximately the same in V*a*. The treatment of the eggs with the indicator at the concentration in question, 3.6×10^{-5} *M*, protects the vitelline membrane against disintegration. Thus the eggs in the test V*b* behave after insemination in normal sea water in a manner similar to that described for e.g. II*b* and IV*b*.

In the tests V*c*–*e* no membrane appeared even if the eggs were fertilized. The lower concentrations of the indicator have thus not been able to protect against the dissolution of the membrane.

The further development was followed in series I–IV. Plutei appeared; in the tests where the eggs had a tighter membrane as in III*b* and IV*b* the plutei were smaller and there was a tendency to dissociation of the cells. In the II series some few fully animalized larvae were observed beside the plutei. The frequency was low (~ 1 per cent); in the control II*a*, however, no animalized larvae were observed.

A parallel experiment with porphyrexid was carried out on the same animal. The results agreed very well with those obtained with porphyrindin.



Eggs of *Psammechinus miliaris* treated for 30 minutes with different concentrations of porphyrindin, then transferred to normal sea water and inseminated. The curve gives the percentage of unfertilized eggs as a function of the concentrations of porphyrindin.

2 B. Fig. 6 gives the result of an experiment with *Psammechinus miliaris*. The eg
were pretreated with porphyrindin for 30 minutes. Thereafter they were transferr
to normal sea water and inseminated here. The counts were made ca. 3 hours aft
insemination.

Some Experiments on the Rate of Fertilization in Eggs Subjected to the Indicators

My colleague Dr. B. Hagström kindly carried out some experiments
which the rate of fertilization was measured according to the method i
troduced by him and Britt Hagström [5]. Eggs of *Psammechinus microtube*
culatus were transferred to a solution of 0.001 per cent lauryl sulphate aft
different intervals until 120 seconds. Thereafter one more determination w
made 1–2 hours after the insemination. The value obtained was regarde
as corresponding to infinite time.

3 A. It follows from the curves in Fig. 7 and the data given in the legends that th
difference is tremendous depending on whether the insemination occurs in the pre
ence of the indicator or after pretreatment. After pretreatment with 5.7×10^{-6} *M*
porphyrindin there seems even to be an increase of rate of fertilization as compare
with the control. In the presence of 7.8×10^{-5} porphyrindin or porphyrexid th
inhibition was practically complete. This result does not seem to depend on a direc
effect on the spermatozoa. These remained motile. Curve 2 is important in this respec
After 120 seconds only 3 per cent of eggs had been fertilized, but in the course of th
following two hours 80 per cent of the eggs became fertilized. No fresh sperm ha
been added, so it must have been the initially added sperm which maintained the
fertilizing capacity. The curves presented are very similar to those which Hagström
and Esping (unpublished) obtained when using a Fucus extract as inhibitor, cf. [17]

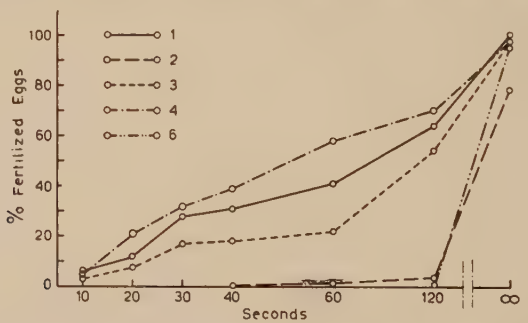


Fig. 7. Percentage of fertilized eggs as a function of time, *Psammechinus microtuberculatus*. (1
Control eggs, inseminated in sea water; (2) inseminated in the presence of 5.7×10^{-6} *M* porphyrin
din in sea water; (3) inseminated in the presence of 5.7×10^{-7} *M* porphyrindin in sea water. (4
Pretreatment of the unfertilized eggs for 10 minutes with porphyrindin 5.7×10^{-6} *M* in sea water
(5) The eggs were inseminated in the presence of 7.8×10^{-5} *M* porphyrexid. No fertilization (n
curve). (6) The eggs were inseminated in the presence of 7.8×10^{-6} *M* porphyrexid. During th
first 120 seconds after insemination practically no fertilization. In the course of 1–2 hours, however
almost 100 per cent of the eggs were fertilized.

Treatment of Fertilized Eggs with the Indicators

development of already fertilized eggs proceeds normally in concentra- f the indicators which are more than 10 times greater than the minimum ntration giving complete inhibition when the insemination occurs in esence of the indicator. Plutei more vigorous than those of the control evelop in concentrations of porphyrexid (e.g. 7×10^{-5} μ) which com- block the fertilization; cf. 1 A and 4 A. Quite similar results were ob- also with porphyrindin.

In one experiment the following test solutions were prepared:

	a	*b*	*c*	*d*
rexid, in sea water	0	1.4×10^{-4}	7×10^{-5}	3.5×10^{-5}
	e	*f*	*g*	*h*
	1.75×10^{-5}	1.1×10^{-5}	5.6×10^{-6}	1.4×10^{-6}

to eleven hours after the transfer the situation was the following:
)0 per cent free swimming blastulae.
lastulae within the membrane, a great part of them presenting dark cytolysis.
lastulae within the membrane or in hatching, very few cytolyzed.
number of free swimming blastulae, the majority within the membrane or in atching.
bout 75 per cent free swimming blastulae, the remainder within the membrane in hatching.
bout 90 per cent free swimming blastulae, the remainder within the membrane in hatching.
)0 per cent had completed hatching, but a smaller number were motionless on e bottom.
s *g*.

i appeared in the test solutions *a–h;* in *b* they were however few and less ed than in *a*. In *c*, *d*, and *e* the plutei were larger and more vigorous than in trol *a; f*, *g*, *h* the plutei were smaller than in the preceding test solutions but re still somewhat larger or at least equivalent to those of the control (observa- . 40 and 65 hours after fertilization).

Details about Incompletely Activated Eggs

nentioned on p. 378, monasters may arise as a consequence of the nation of eggs subjected to a prolonged pretreatment of the eggs with licators. A closer examination revealed that the monasters were the

consequence of an incomplete fertilization of the eggs. In several cases it turned out that the spermatozoon is unable to pass through the membrane covering the egg. The mere attachment of the spermatozoon suffices to give the egg an activation. An incomplete membrane formation occurs, the nucleus migrates to the centre of the egg and a monaster develops. Sometimes this has a vortex-like appearance which must be due to rotating movements in the cytoplasm.

In one experiment the eggs had been pretreated with 7.4×10^{-5} *M* porphyrindin for 17 hours. They were then washed in sea water and inseminated an hour later. About 50 per cent of the eggs remained non-activated; the others were activated and cleavage followed within a rather tight membrane the normal elongation of the egg at first telophase failed to occur. After the first cleavage the blastomeres mostly appeared as two half-spheres tightly pressed against each other, whereas in the normal control eggs the surface of contact between the first two blastomeres was smaller and the radius of the curvature of the cell surfaces decreased toward the cleavage plane. In 15–20 per cent of the whole material, however, no cleavage occurred: a gamut of different cases of incomplete activation was found here. Fig. 8 illustrates the case where a spermatozoon was attached to the membrane. This was elevated only in the region of sperm attachment. Here the membrane appeared to be of normal thickness whereas outside this region the membrane was thin. No other spermatozoon was attached to the membrane and no spermatozoon had penetrated into the interior, where the monaster was formed. The monaster formation did not lead to spindle formation and cleavage.

The tail of the spermatozoon attached to the membrane was motile still 4 hours after insemination.

In Fig. 9 another similar case is represented but the membrane was rather low even in the region of sperm attachment.

The reaction against an attached but not penetrating spermatozoon may be still more localized than in the previous cases. A concavity arose for example

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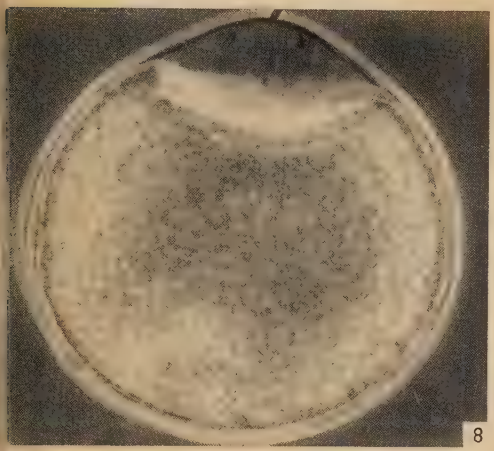
Figs. 8–13. *Paracentrotus lividus*. Different cases in which a spermatozoon has attached itself to the surface of the egg which had been pretreated for 17 hours with 7.4×10^{-5} *M* porphyrindin in sea water. Thereafter they were transferred to normal sea water and inseminated here. In *Fig. 8* rather strong local membrane formation. Monaster formation in the interior. *Fig. 9*. The membrane was less elevated even in the region of attachment of the spermatozoon. *Fig. 10*. More localized action of the attached spermatozoon. The interior had the structure of an unfertilized egg. *Fig. 11*. A case similar to that of Fig. 10. The membrane certainly expanded over a larger region but the interior had rather the character of an unfertilized egg. In *Figs. 12* and *13*, the spermatozoon had also a localized action but a thin membrane extended over the surface of the egg. A monaster appeared in the interior of the egg. Photos about three hours after insemination. Phase contrast.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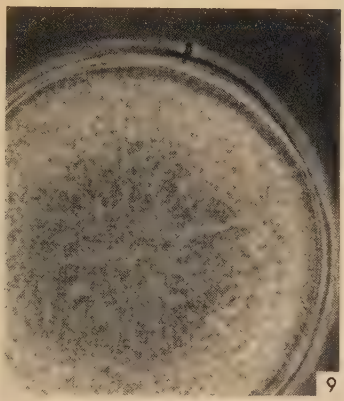
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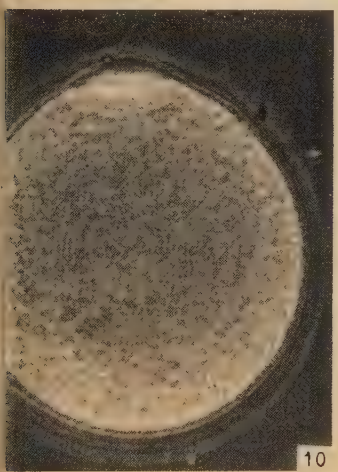
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12

13

and a membrane appeared above the concavity as shown in Fig. 10. A spermatozoon was attached to the membrane. It had caused an expulsion of cortical granules and their incorporation in the membrane as was indicated by its thickness but evidently the impulse had soon died off. The main part of the egg had the characteristic coarser structure of an unfertilized egg. The optical properties of the cortical granules indicated in they were still enclosed in the cortex. The nucleus had an eccentric position and presented no increase of volume. Fig. 11 represents a similar case but the membrane had separated over a somewhat larger area.

In one case a spermatozoon was observed where the head was not perpendicular but lying parallel to the egg surface. In a region with a radius 3 × the length of the head of the spermatozoon the cortical granules had apparently disappeared, i.e. they were incorporated in a membrane. In the rest of the egg the cortical granules were still present in the cortex but they were more clearly visible in phase contrast than is usually the case in non-activated eggs. They were probably under way of expulsion from the cortex but the incorporation into a fertilization membrane had not occurred. Figs. 12–13 give another two cases in which the spermatozoon has been trapped in the egg surface. Although the membrane is low even in the region of sperm attachment it extends over the whole egg; a monaster formation has occurred.

Monaster eggs may arise also when the spermatozoon penetrates into the interior of the eggs. It could be made out in several cases that the sperm nucleus and middle piece were present within the egg but no spermaster developed. Instead a monaster was formed around the female nucleus. The difference is that in these cases the spermatozoon was able to cause a local cortical reaction but the normal interaction between cortex and spermatozoon was not brought about. Without this interaction no spermaster develops [21]. On the other hand even a local activation of the cortex causes a monaster formation. Dissolution and reformation of the nuclear membrane and a growth of the whole nucleus were observed.

A monaster formation may occur even if the interior cytoplasmic structure is of the "unfertilized" type provided that at least a local activation of the cortex has occurred.

Attachment of a spermatozoon without penetration may cause the formation of a concavity without membrane elevation. The peripheral cytoplasm showed a tangential orientation which is most prononuced in the part adjacent to the concavity. The orientation may indicate a movement of the cortical cytoplasm in the direction of the concavity.

Effect of the Indicators on the Oocytes

Numerous spermatozoa may enter the oocytes which have no mechanism protection against polyspermy. As described already by Wilson [23] mous papillae form on the surface of oocytes which have been insemi- They represent enlarged entrance cones.

such eggs the spermatozoon remains in the periphery of the egg and develops an aster. These facts point to a great difference of constitution between the cortex of the oocyte and that of the mature egg. This difference early emphasized by Wilson [24] is also demonstrated by observations on and eggs immersed in solutions of the indicators tested here. Concentrations of the indicators which completely block fertilization allow the formation the papillae.

In one experiment with *Paracentrotus lividus* the eggs were pretreated for an with porphyrindin in sea water and then inseminated in the solutions containing indicator. The concentrations were the following:

	a	*b*	*c*	*d*
Porphyrindin, *M* in sea water	0	2.14×10^{-5}	1.16×10^{-5}	2.9×10^{-6}

a–*d* the oocytes formed papillae upon insemination, whereas the fertilization mature eggs was completely blocked in *b*, almost completely in *c* and not blocked and *d*. In *d*, however, the fertilized eggs show certain subnormal traits. In concentrations higher than those indicated above the reactivity of the oocytes is lowered fails to occur. In an experiment with porphyrexid the oocytes showed still formation of small papillae in the presence of 3.5×10^{-5} *M* of the indicator. In 10^{-4} *M* no changes of the oocytes were noted although the spermatozoa exhibited lively movements.

The critical concentrations blocking the formation of the papillae may be different in oocytes of *Paracentrotus lividus* and those of *Psammechinus miliaris*. In the latter species the presence of 10^{-4} *M* porphyrindin blocked the fertilization completely whereas the oocytes reacted upon the insemination formation of numerous papillae. These gradually retracted but if the eggs were fixed in the solution of porphyrindin the oocyte was divided into two parts one larger containing the nucleus and one smaller. The spermatozoa which from the beginning were distributed over the entire surface of the oocyte grew concentrated to the smaller part. Evidently this represents the fertilization layer, the main part of which under certain circumstances may be pulled off from the surface of the egg. Sometimes the oocytes were subdivided into three parts of which the middle one represented the cortex.

A similar contraction of the cortex was earlier found to occur under th action of periodate [19].

These observations again underline the great difference between the prope ties of the cortical layer of the oocyte and that of the mature egg. In this latte a somewhat similar behaviour has only been observed in *Arbacia* eggs sub jected to parachloromercuribenzoate [20].

Effect of the Indicators on the Jelly Coat

The indicators also influence the jelly coat surrounding the egg. Mor spermatozoa are collected in the jelly coat in the presence of e.g. 3.6×10^{-5} porphyrindin than in that of control eggs of *Paracentrotus lividus*. Experienc has shown that this is a sign of a higher rigidity of the jelly coat in the eg exposed to the indicator. When the eggs were kept between coverslip an slide in a suspension of china ink in sea water the jelly coat did not seem more swollen in the control eggs than in those subjected to porphyrindin However, when a light pressure was repeatedly exerted on the coverslip the jelly coat of the control eggs was removed. Conversely the jelly coat of th eggs subjected to porphyrindin proved to be resistent to the same treat ment. The effect on the jelly coat resembled that exerted by fertilizatio inhibitor from *Fucus vesiculosus*, which has previously been reported [17 It cannot be decided as yet whether the change of rigidity of the jelly coa occurs as a direct or as an indirect effect of the action of the indicators.

The Non-inhibitory Effect of Porphyrexin

The formula of porphyrexin was given above (p. 374, I). It represents the reduced form of porphyrexid (p. 374, II). Porphyrexin proved to be inactiv as a fertilization inhibitor.

6A. Porphyrexin was dissolved in sea water. In one experiment with eggs from *Psammechinus miliaris* the following solutions were tested:

a	*b*	*c*	*d*
Sea water	6.4×10^{-3}	3.4×10^{-3}	1.3×10^{-3} *M*

The eggs were treated for 26 minutes with the porphyrexin solutions and then inseminated while still exposed to the solutions.

A membrane was elevated in normal way in all the solutions. All the oocytes showed a very rich formation of papillae.

development of the eggs which were left in the porphyrexin solutions lead tulae with a normal blastocoel in all the solutions. A slight influence was ex- the highest concentration of the substance on the plutei. These presented at shorter arms in the concentration 6.4×10^{-3} *M* than in the control, *a.* ower concentrations tested the plutei resembled those of the control.
lution of porphyrindin 10^{-4} *M*, i.e. 1/64 of the highest concentration of rexin used in the experiment, gave under the same conditions almost 100 per hibition of fertilization.

e Effect of Glutathione after Previous Treatment with Porphyrindin

nmechinus miliaris eggs were pretreated with porphyrindin (10^{-4} and 0^{-4} *M*) and were washed in sea water. Thereafter a portion of the eggs ansferred to 0.025 per cent glutathione in sea water. The solution was t to the pH of the sea water. After e.g. 10 minutes the eggs in glutathione vashed. The treatment of the eggs with glutathione seemed to remove hibitory effect of porphyrindin to a certain extent. Often it was not s that the membrane was more elevated as a consequence of the ex- to glutathione but the membrane appeared softer. It offered less nce to the elongation of the egg previous to the first cleavage. In ases, however, a more obvious improving effect was noted. Even when provement of the membrane elevation was slightly marked the per- of developing eggs was considerably lower after treatment with por- din alone than after a subsequent exposure to glutathione.

Survey of treatment of the eggs *(Psammechinus miliaris):*
a water.
5×10^{-4} *M* porphyrindin in sea water for 40 minutes. Thereafter the eggs ere washed in sea water (dilution factor 10^3).
ggs exposed to porphyrindin and washed as under *b*. Subsequently they were red to 0.025 per cent glutathione for 10 minutes and then washed in sea dilution factor 10).
were inseminated after the last washing of *c*. The result was the following:

No membrane elevated per cent	Membrane partly elevated, eggs angular per cent	Membrane elevated, egg surface smooth per cent	*n*
—	—	100	>200
68	22	10	95
25	20	55	102

bout 50 minutes the first division started in *b;* the daughter cells were more ssed than in *c*, where they had a greater capacity to round up. This was due reater restraint which the low membrane exerted on the eggs in *b*, cf. Figs. 14

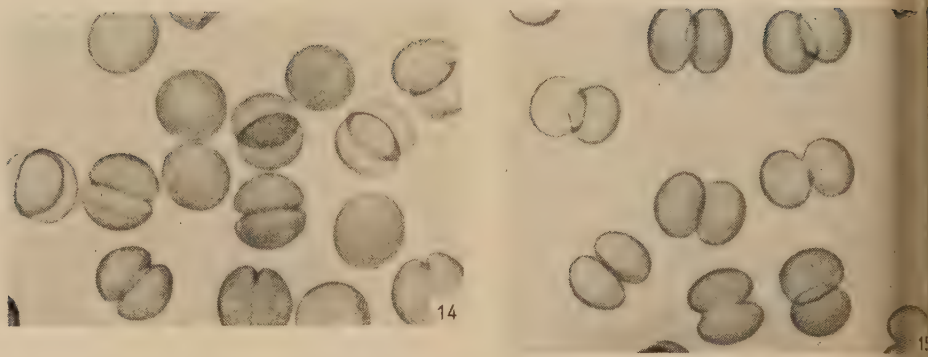

Fig. 14. Eggs of *Psammechinus miliaris* pretreated for 40 minutes with 2.5×10^{-4} *M* porphyrindi in sea water. Inseminated after transfer to normal sea water. Ca. 60 minutes after fertilization. Fig. 15. The eggs had been pretreated as the eggs of Fig. 12, but instead of being transferred fro porphyrindin directly to normal sea water the eggs were subjected to 0.025 per cent glutathion in sea water and only thereafter inseminated in sea water with a lowered conc. of glutathion cf. exp. 7A.

Removal of the Porphyrexid Effect on Fertilization by Addition of α-Lipoic Acid

The oxidized form of α-lipoic acid was added in some experiments alon with porphyrexid. By addition of certain quantities of α-lipoic acid the effec of the indicator on fertilization can be suppressed entirely.

8A. The following molar solutions were used in the tests:

	a	*b*	*c*	*d*
Porphyrexid	0	4.2×10^{-5}	0	4.2×10^{-5} *M*
Oxidized lipoic acid	0	0	2.2×10^{-3}	2.2×10^{-3}

Ten minutes after transfer of unfertilized eggs to the test solution they were in seminated:

2 minutes after insemination:

a. 100 per cent fertilization with well-elevated membranes.
b. No fertilization.
c. 100 per cent fertilization with membranes even somewhat higher than in *a*.
d. 100 per cent fertilization. 95 per cent of the eggs with membranes somewhat lower than those in the control; 5 per cent with membranes equal to those in *a*.

6 hours, 45 minutes after insemination:

a. Blastulae with large blastocoel.
b. Unfertilized eggs without sign of activation.
c. Development somewhat delayed; there is a pronounced tendency for dissociation of the cells.
d. Rather similar to *c*.

hen fertilized eggs were immersed in 4.2×10^{-5} *M* porphyrexid, e.g. 10 minutes t insemination, they developed but the blastocoel was smaller than in the con- o although the diameter of the blastulae was about the same as in the control. out 7 hours after insemination the embryos in *a–d* of the experiment referred re transferred to normal sea water. The tendency for dissociation of the cells a ow overcome. In *c* and *d* animalized larvae developed.

Reduction of Porphyrindin by the Unfertilized and Fertilized Eggs as well as by α-Lipoic Acid

e blue porphyrindin solution was reduced by the eggs. The rate was g r in fertilized than in unfertilized egg. The quotient of the rate of reduc- o n the fertilized over that of unfertilized eggs parallels rather closely the u ent between the rates of respiration. Porphyrindin proved to be reduced s taneously by the reduced form of α-lipoic acid.

. Equally concentrated suspensions of unfertilized and fertilized eggs were r ht into solutions of porphyrindin in sea water. The final concentration of the d tor was 9.2×10^{-5} *M*.

eady after 20 minutes a decoloration of the suspensions was visible when they e compared with a blank containing only porphyrindin.

er 3 hours and 40 minutes the extinctions at the wavelength 580 mμ were as l s:

Blank with porphyrindin	0.175
Suspension of unfertilized eggs	0.080
Suspension of fertilized eggs	0.030

phyrindin and the reduced form of α-lipoic acid were mixed so as to make the n concentrations 9.2×10^{-5} and 6.8×10^{-4} *M* respectively. The reduction was s taneous.

DISCUSSION

is paper demonstrates that the effect of the two high potential oxidation d tion indicators porphyrexid and porphyrindin on the ripe sea urchin g very different from that of sodium periodate. Their main points of attack u thus be rather different. Periodate oxidizes mainly α-glycol groups in a hydrates and proteins. Porphyrexid and porphyrindin act in virtue of e oxidizing properties as follows from the fact that the reduced form of o yrexid, the porphyrexin, proved to be harmless to the eggs. In the s place SH-groups are oxidized by the indicators.

e solidification of a layer in the egg surface should thus be due to oxida- o f SH-groups which may lead to an increased polymerization of poly-

peptide chains, respectively to an inhibition of enzymes in which free SH groups are prerequisite for activity. This view seems to be contradicted by the fact that glutathione gives no complete reversal of the effect of porphyrindin. Several reducing agents should be tested but it can already be stated that not necessarily all the cystine linkages in a protein are easily accessible for reducing agents, cf. Farnworth [4]. It seems quite possible that an oxidation through the high potential indicators brings about similar conditions. The effect will of course depend on the concentration of the indicators and the duration of the treatment.

The observed effects of porphyrexid and porphyrindin are indicative of changes in the surface layers which occur at maturation and fertilization of the egg. The oocyte reacts upon insemination by formation of papillae in the presence of porphyrindin in concentrations which block the fertilization of the mature egg. The fertilized eggs sustain the action of the indicators better than the unfertilized mature eggs.

The eggs reduce the indicators, thus converting them into the harmless reduced state. This process is more rapid in fertilized than in unfertilized eggs. The oocytes probably behave as the fertilized eggs, cf. [12]. This may be one reason why the fertilized eggs and the oocytes are less sensitive than the unfertilized mature eggs.

If the unfertilized mature eggs are inseminated in the presence of the indicator there is a complete block to fertilization down to the concentration of about 5×10^{-6} *M*.

Experiments 2A and 2B show that the block is reversible at least as far as the cytoplasm of the egg is concerned, provided the eggs were not subjected to too high concentrations of the indicators or the duration of the exposure was too long, cf. e.g. exp. 2A, I*b*. The pretreatment changes, however, the most superficial layer of the egg which is involved in the formation of the fertilization membrane.

It seems probable that the layer directly affected is the vitelline membrane, a thin metaplasmatic structure covering the surface of the cortex.

The existence of a vitelline membrane has been disputed, cf. [12]. Yet Chambers [3] gave already, by micromanipulation, evidence of the existence of this membrane in unfertilized sea urchin, sanddollar and starfish eggs. Recently Afzelius [1, 2] has convincingly shown by electron microscopy that there really exists a membrane covering the cortex. This membrane is 100 Å thick and corresponds to the vitelline membrane. As the fertilization impulse spreads over the egg the cortical granules join the vitelline membrane. In connection with the elevation of the membrane they merge with the vitelline

brane, thus increasing the thickness of the elevating membrane to t 500 Å, cf. also [10]. In the older literature the elevated fertilization brane was often called the vitelline membrane. It is however more opriate to reserve this name for the delicate membrane covering the x of the unfertilized sea urchin egg. In eggs of sanddollar and starfishes membrane is evidently tougher.

eggs of *Echinocardium cordatum* or *Psammechinus microtuberculatus* the vation was made that an extremely thin membrane appears upon fer- ion. This membrane is connected with the egg surface by means of thin ds; for references see [13, 14]. The membrane rapidly becomes thicker nore refractile. Earlier the writer considered that the "strands" correspond matrix in which the cortical granules are embedded. New observations however, led to the opinion that the strands are in fact strongly stretched al granules. These were often observed also in eggs fertilized after pre- ment with porphyrexid or porphyrindin, cf. Figs. 3–5.

was found that the pretreatment with the indicators makes the sperm nce more difficult. This must be due to the toughening of the exterior ce of the cortex. Moreover this toughened layer is elevated upon fer- ion. Consequently this layer must correspond to a continuous vitelline brane. This problem will be further elucidated in a following paper [15].

e effect of the indicators is more lasting within metaplasmatic structures within the cytoplasm. This seems reasonable. The role of the metabo- of the latter has already been pointed out. Metaplasmatic structures, like itelline membrane, are devoid of a metabolism of the same extent.

e removal of the effect of the indicators by means of α-lipoic acid could bly mean that this easily diffusible substance serves as a link between etaplasmic structures in the cell surface and the metabolic systems in terior.

e observations indicate that even deeper layers of the cortex than the ne membrane are involved in the action of the indicators persisting after transfer to normal sea water. As already pointed out the cortical les are often stretched between an elevated part of the membrane and gg surface. This means that the inner part of the cortical granules still es to the egg surface. In the normal fertilization the cortical granules t adhere to the egg surface but only to the vitelline membrane. An in- ed fluidity in the cortex layer arises upon fertilization which allows the les to move toward the vitelline membrane and attach themselves to membrane. No inner connection with the cortex can be demonstrated. oxidation has occurred subsequent to the treatment with indicators the

increased fluidity is suppressed by oxidative polymerization or inactivation of SH-enzymes with the consequences previously described.

This leads to the conclusion that reduction of cystine linkages may play a role in the cortical changes resulting in the release of the cortical granules.

It has been found that thioglycolate decreases the rigidity of the cortical layer. At the same time it facilitates the fertilization and the elevation of the fertilization membrane, cf. [23]. A number of proteins and amino acids act in the same way and their effect seemed rather enigmatic. Some light will be shed perhaps from the recent observations by Kozloff and Henderson [7] and by Kozloff [5]. According to them complexes between amino acids or proteins on the one hand and divalent metals Zn^{++} or Cd^{++} on the other bring about a splitting of the distal part of the tail in bacteriophage T^2. The proteins may also be replaced by cyanide. There is evidence that the effect of the metal complexes is to rupture disulfide bonds present in the protein structures of the distal part of the tail. A similar effect can be exerted by thioglycolate. It is quite possible that a comparable mechanism is operating in the surface of the sea urchin egg subjected to solutions of amino acids or proteins. These may form complexes with divalent ions present in the egg cortex. Cyanide is also a potent agent for changing the surface of the non-fertilized ripe egg towards a higher capacity of membrane formation in a hypertonic medium [22].

Reduction of disulfide bonds may be involved in cytoplasmic maturation. The egg cortex may be richer in these bonds immediately after the maturation but gradually they may increase in number. The treatment with thioglycolate or with proteins or amino acids removes immediately the state of underripeness. Treatment with periodate does the same but the mechanism is here a different one.

It has been shown that a prolonged pretreatment of the egg with the high potential indicators decreases the impulse so that only a part of the egg may be activated; cf. Figs. 8–13. This speaks in favor of the conclusion that SH-groups or at least absence of certain disulphide bonds are necessary for the propagation of the impulse. It was demonstrated earlier [20] that the presence of SH-reagents even in rather high concentrations do not interfere with the attachment of the spermatozoon and the spreading of the impulse. It seems to be contrary to the conclusions expressed above that even iodosobenzoate does not block the attachment of the spermatozoon and the spreading of the impulse. This apparent contradiction cannot be resolved at present but the high potential indicators are probably much more efficient oxidizing agents than is iodosobenzoate.

SUMMARY

The effect of oxidizing agents on the sea urchin egg has been studied previously [9, 16, 19]. The promoting effect of periodate on the fertilization and unique among a number of oxidizing agents tested. In the present the effect of the two high potential oxidation-reduction indicators, yrindin and porphyrexid, has been studied. In the presence of these is a block to fertilization down to concentrations of about 5×10^{-6} *M*. block to fertilization is reversible at least as far as the cytoplasm of g is concerned, provided the concentrations of the indicators were o high or the duration of the exposure too long, cf. exp. 2A.

effect of the indicators on metaplasmatic structures in the egg surface vitelline membrane is non-reversible or much less reversible than that cytoplasm. This is referred to the lack of metabolic processes in the lasmatic structures, whereas in the cytoplasm the indicators will be ted into their harmless reduced forms.

effect of the indicators can be removed to a certain extent by subseexposure to reduced glutathione; α-lipoic acid was found to counteract uction in a still more pronounced way.

n deeper layers of the cortex than the vitelline membrane are involved action of the indicators persisting even after transfer to normal sea The cortical granules were often found to remain attached to the asmic surface. Upon (partial) elevation of the membrane the attached es may become stretched, cf. Figs. 3–5.

suggested that reduction of disulfide bonds may be involved in cytoic maturation. The effect of substances like thioglycolate [22] on the y of the cortex is briefly discussed.

surfaces of oocytes and fertilized eggs are less sensitive to the oxidizing tors than are those of the unfertilized mature eggs.

solidification of the vitelline membrane under the action of the inrs may remain a hindrance for sperm entry even since the eggs have rought into normal medium. It was often observed that a spermatozoon es itself to the egg surface but failed to penetrate. Nevertheless the atozoon is able to start an impulse which spreads over the egg surface. rface area covered by the impulse may be small. This leads to a highly ed membrane formation. The main part of the interior cytoplasm may in the unfertilized state. A monaster may, however, often be formed the nucleus even following a rather incomplete activation. The imnay also cover a large area or the whole egg surface. The membrane is

often elevated only around the site of attachment of the spermatozoon. This must mean that the impulse has suffered a decrement, resulting in a decrease of the number of expelled cortical granules.

Concavities are often formed on the egg surface subsequent to attachment of a spermatozoon.

Even if a spermatozoon penetrated through a toughened surface into the interior of the egg a sperm aster sometimes failed to develop but a monaster formation could occur.

This work was supported by grants from the Swedish Natural Sciences Research Council and the Swedish Cancer Society, which is gratefully acknowledged.

I am greatly indebted to Dr. Peter Dohrn and Dr. Gunnar Gustafsson for their ready helpfulness during my sojourns at the Zoological Station, Naples, and Kristineberg Zool. Station.

I want also to express my most sincere thanks to Dr. Hans Bielig, Heidelberg, for his kindness in providing the valuable substances, porphyrexin, porphyrexid and porphyrindin. Likewise I am greatly obliged to Dr. Enrico Cutolo, Istituto Sieroterapico Italiano, for generous repeated gifts of α-lipoic acid.

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BRIEF NOTES

UGATION OF THE HETEROPYCNOTIC X AND Y CHROMOSOMES OF THE RAT SPERMATOCYTE

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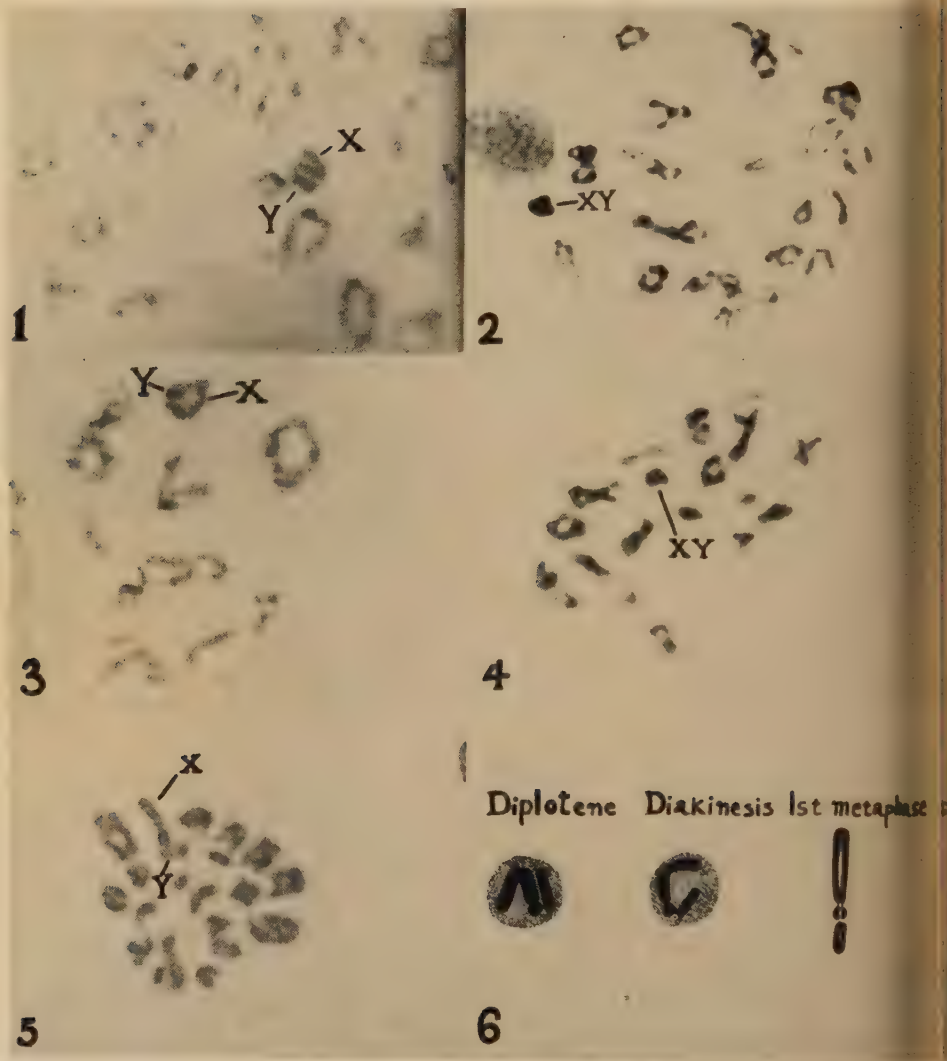
XY bivalent of rat primary spermatocytes has been shown to be heteropyc- uring meiotic prophase [2, 3] and to be embedded in a vesicle rich in RNA [2]. dividualities of the X and the Y are concealed in this state. Accordingly, their of conjugation is obscure, even during diplotene. Recently, by means of pre- ent with distilled water, which permits a wide spreading of the chromosomes, stion with ribonuclease, it has been possible to unveil the individualities of and the Y in diplotene as well as in early diakinesis so that it may be possible ify this point concerning the presence or absence of cytological crossing-over n the X and the Y in the rat.

ar rats were used for the study. Excised fresh seminiferous tubuli were handled of three ways. (1) They were minced and washed in a drop of distilled water on a microscopic slide. Liberated cells were allowed to stand for 20 seconds in nic suspending fluid and then an equal volume of glacial acetic acid was The cells were squashed between the slide and a coverslip; then they were by the Feulgen method. (2) For digestion of the RNA material, a ribonuclease n, containing 10 mg recrystallized Armour ribonuclease in 100 cc of physio- saline, was used. The cell suspension fluid was aspirated into a blood-count and incubated for 15 minutes at 37°C. Following the incubation, the squash n procedures were carried out as usual. (3) For comparison, ordinary squash ations were made by using physiological saline without any treatment preced- Feulgen method.

ither of the mentioned special methods using water or ribonuclease solution, nct picture of the heteropycnotic XY in late diplotene was demonstrated 1, 6). The X was bent at the middle in a V-shaped form. The bending takes with no relation to the kinetochore which in the case of the X chromosome is subterminally. Therefore, the observed V-shape is not analogous to the V- chromosomes observed during metaphase. The Y was always found lying parallel to one portion of the bent X; that in which the kinetochore is located. lationship strongly suggested a side-by-side conjugation and, possibly, the gous nature of these portions. Several chromatin strands were often found n them. These may be artefacts. In the ordinary squash preparation, the XY stage was found as a heteropycnotic mass without showing structural details).



Photomicrographs of the rat primary spermatocytes in first meiosis, taken by Leitz Panphoto n 15 × 90. X: X-chromosome; Y: Y-chromosome; XY: XY bivalent.

Fig. 1. Late diplotene in the preparation made by the distilled water method. Individualities of the X and the Y are seen.

Fig. 2. Late diplotene in the ordinary squash preparation. The XY is a heteropycnotic body.

Fig. 3. Early diakinesis in the preparation treated with ribonuclease. The heteropycnotic X and Y may be seen to be in end-to-end relationship.

Fig. 4. Early diakinesis in the ordinary squash preparation. The XY is still a heteropycnotic body.

Fig. 5. First metaphase in the ordinary squash preparation. The XY is isopycnotic to the autosomal tetrads. The Y is connected with the X end-to-end.

Fig. 6. The structural arrangement of the XY, changing during first meiosis, is schematically illustrated. The vesicle, rich in RNA, in diplotene and diakinesis is designated by the shaded area.

arly diakinesis, the relationship between the two chromosomes has changed. ow maintain close contact merely at their ends. Similar relationship of the X e Y has been demonstrated by Tjio and Levan [3]. In ordinary squash prepara- only a deeply staining heteropycnotic body indicating no individual chro- es has been observed (Fig. 4). In late diakinesis, the X and the Y become otic to the autosomal bivalents. They are connected end-to-end, as stated and ted in Fig. 5 in the previous paper [2]. In first metaphase, the X and the Y e observed to be associated end-to-end (Figs. 5, 6). This has been observed by workers [1, 3].

present study appears to have demonstrated the serial changes in relationship n the X and the Y during meiosis. The side-by-side localization of the Y and rtion of the X at diplotene suggests a possibility of the presence of cytological g-over and the exchange of genetic materials between them.

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OME OBSERVATIONS ON PINOCYTOSIS IN LEUCOCYTES

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estigations on rabbits, Pentimalli [11] has demonstrated that repeated ons of foreign proteins may give rise to a leukemic condition. Demonstration presence, within tissue cells, of parenterally administered proteins [1, 4, 7] interest on the mechanism by which such proteins enter the cells, and on the n of which cells are concerned with uptake.

is [2] has reported the occurrence of pinocytosis in leucocytes from blood; he es the cells as taking up vacuoles of fluid by enveloping droplets in the pe- l membrane and passing them to the centre of the cell. Bessis does not state dium in which he observed pinocytosis, which he regards as a degenerative . The "Sekretblasen" described by Franke [8] in leucocytes and in myeloblasts to be pinocytosis vacuoles.

rimental. Material and methods.—Leucocytes from peritoneal exudates of pig and rabbit were prepared by injecting "Bacto" bacteriological medium e peritoneal cavity of the animals, which were killed 15-20 hours later. The

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Fig. 1. Phase contrast photograph of living leucocyte in dilute (less than 1 per cent) solution of beta-lactoglobulin. Newly formed pinocytosis vacuoles can be seen at the periphery of the cell. Total magnification × 2000.

abdominal cavity was opened immediately and the leucocytes washed out with sterile physiological saline. After centrifugation in an angle-head clinical centrifuge for three minutes at ca. 1000 r.p.m., removal of the supernatant fluid, resuspension in fresh saline, and repetition of this process twice, the leucocytes were examined at room temperature (17–23°C) to control viability, using a Galileo phase contrast microscope, objective 100×, total magnification 1000×. Clean thin white slides and thin coverslips were used, the latter being sealed with molten paraffin to prevent evaporation.

Solutions of the following proteins were used: bovine gamma globulin (Armour and Co.), human serum albumin (Serum Institute, Copenhagen), crystallized bovine plasma albumin (The Armour Laboratories), human serum albumin (prepared at Istituto Regina Elena), and beta-lactoglobulin (The Armour Laboratories). The proteins were dissolved in sterile physiological saline, immediately before use; concentrations ranged from 1 to 4 per cent. When not under observation the leucocytes were kept in stoppered glass tubes at 37°C. Sterile capillary pipettes were used for all transfers of cells and solutions.

Results. Repeated phase contrast observations were made on washed, unseparated leucocytes in physiological saline. These cells regularly showed the phenomenon designated "étalement" by Bessis and Bricka [3], the cells flattening out and creeping slowly on the glass surface of the slide. The cells constantly changed shape and movement of the intracellular granules was pronounced; under these conditions the cells remained motile for at least a day. After 6 hours or more the cells occasionally showed the perinuclear vacuolization described by Bessis [2], but they were never observed to take up vacuoles from the surrounding medium.

leucocytes were placed in 1–4 per cent solutions of the various proteins, it served that they did not show "étalement" but remained contracted and spherical. Formation of vacuoles at the periphery of the cells commenced a few minutes of immersion, and corresponded to that reported by Bessis [2], s of fluid being enveloped by the peripheral membrane. The process of vacuole on was rapid, only a few seconds being required for completion. The cells replete with vacuoles and ceased pinocytosing at intervals; extrusion of s was never observed. In these preliminary experiments, the observations p to 6 hours, at which time the cells contained many small vacuoles, and med fresh vacuoles at intervals; there were no signs of degeneration.

minimal concentration of protein required to produce pinocytosis was not ned, but it is certainly less than 1 per cent, as was shown in experiments h a slide-coverslip preparation of washed leucocytes was made, but not After the cells had "etaled", a small drop of a 1 per cent solution of beta- bulin was introduced at one corner of the coverslip. After a few minutes the tes started to form vacuoles, at the same time retracting to a spherical shape. ells continued to pinocytose intermittently for at least 6 hours. Phase contrast aphs of the cells were taken at intervals of 5 minutes, and showed the con- changing periphery of the cells and the formation of vacuoles. Fig. 1 is a aph from this series. The cells were flattened slightly by gentle pressure on erslip to facilitate photography, and hence do not show the typical spherical characteristic during pinocytosis.

e cell types encountered in the exudates showed pinocytosis and all the pro- fered produced the same effect, but more pronouncedly in solutions of gamma s and beta-lactoglobulin than in those of albumins. Some experiments, which reported later, using ^{131}I labelled proteins, in which measurements were made uptake of protein by the cells after varying periods of pinocytosis, also in- that solutions of albumins were less attractive to the cells than those of s; this is interesting in view of the fact that it is the globulin fraction of the which contains antibodies [4].

us appears that, in analogy to other amoeboid cells [5, 6, 9, 10], leucocytes able, at least *in vitro*, of taking up solutions of proteins from the surrounding ; hence the possibility that this may be a normal method for the entry of proteins into the cell cannot be ignored.

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OBSERVATIONS AU MICROSCOPE ÉLECTRONIQUE SUR LES MODIFICATIONS DU CYTOPLASMA AU CONTACT DES PARTICULES DE SILICE COLLOÏDALE

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Au cours de recherches sur la pathogénie des pneumoconioses nous avons été amenés à étudier au microscope électronique la phagocytose, par les cellules alvéolaires du poumon, de particules de silice colloïdale introduites par injection intratrachéale. La présente note envisage les réactions submicroscopiques du cytoplasma au contact de ces particules.

Technique.—Des rats reçoivent 20 mg de silice colloïdale particulaire par voie intratrachéale. Après un délai variable, les animaux sont narcosés au Nembutal, des fragments de poumons fixés au Palade tamponné isotonique à + 2°C. Inclusion au méthacrylate de butyle prépolymérisé avec du peroxyde de benzoyle à 1 %. Les coupes sont effectuées au microtome de Porter Blum et examinées au microscope RCA EMU 3 A.

Resultats.—Les résultats observés montrés par les figures ci-jointes, peuvent être résumés ainsi.

Les particules (150 à 1000 Å environ) sont groupées en petits agglomérats d'importance très variable (quelques unités ou plusieurs centaines de particules). Ces agglomérats sont toujours logés dans la matrice cytoplasmique fondamentale, jamais dans l'intérieur même des éléments du réticulum endoplasmique, vésicules, canalicules ou sacs aplatis.

Autour de ces amas particulaires, dans un rayon très faible, de 0,1 à 0,2 micron en général, le cytoplasma apparaît dense et homogène, sans granulations de Palade.

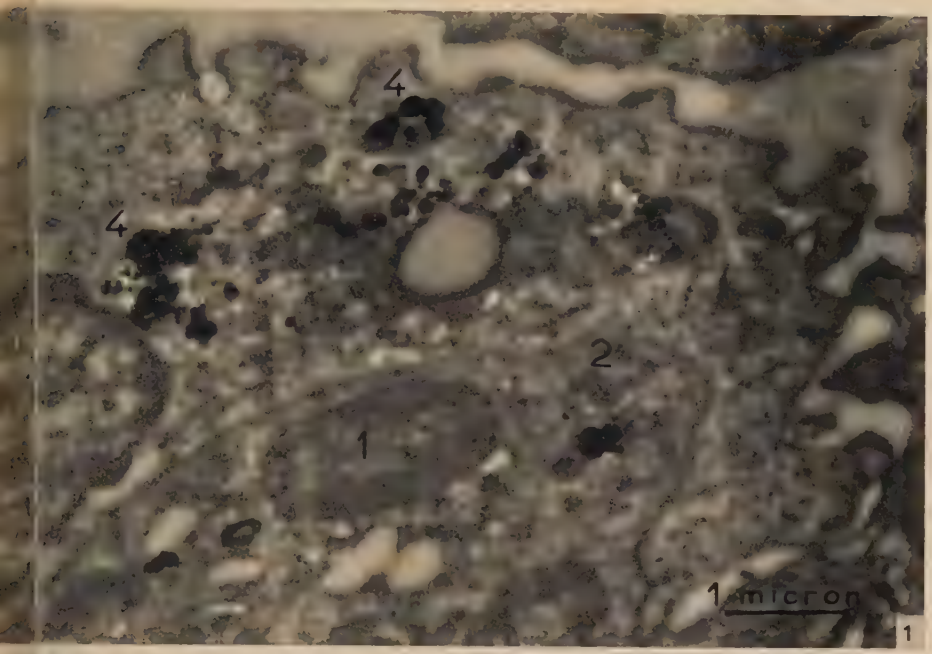
Au voisinage immédiat des agglomérats de particules on observe très fréquemment de petites vacuoles à contenu très peu dense, souvent fort clair. Certaines de ces vacuoles doivent certainement représenter le logement de particules arrachées au moment de la microtomisation. Par contre, d'autres vacuoles plus irrégulières et moins claires semblent devoir être rapportées à des altérations du cytoplasma fondamental. Ces vacuoles, dépourvues de membrane périphérique, ne semblent pas pour cette raison appartenir au système du réticulum endoplasmique.

On n'a pas pu constater jusqu'à présent de rapports particuliers et constants des amas de particules avec les mitochondries. Ils peuvent se trouver quelquefois

Légende générale : *1* noyau cellulaire; *2* mitochondries normales; *3* mitochondries altérées; *4* agglomérats de particules.

Fig. 1. Phagocyte pulmonaire (Rat), injection intra-trachéale de silice colloïdale, examen après 4 jours. Les particules sont unies et leurs limites obscurcies par une substance dense et homogène. Grossissement : 13,000 ×.

Fig. 2. Lésions pulmonaires, Rat, 4 jours après injection de silice. Au niveau des particules de silice se voient des vides artificiels résultant de l'arrachement de particules pendant la microtomisation. Le cytoplasme entre les agglomérats est densifié. Grossissement : 13,000 ×.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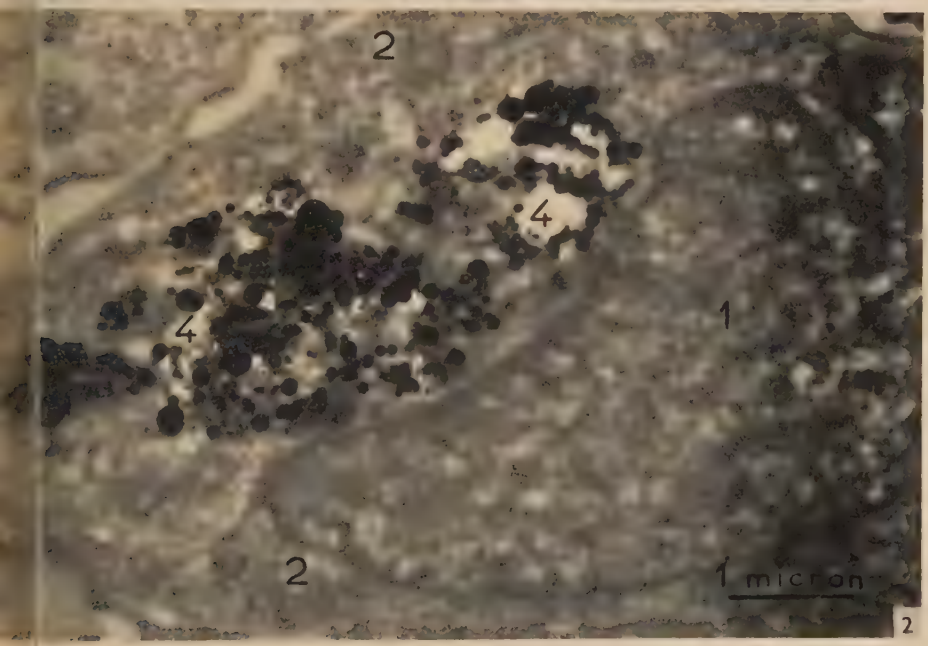
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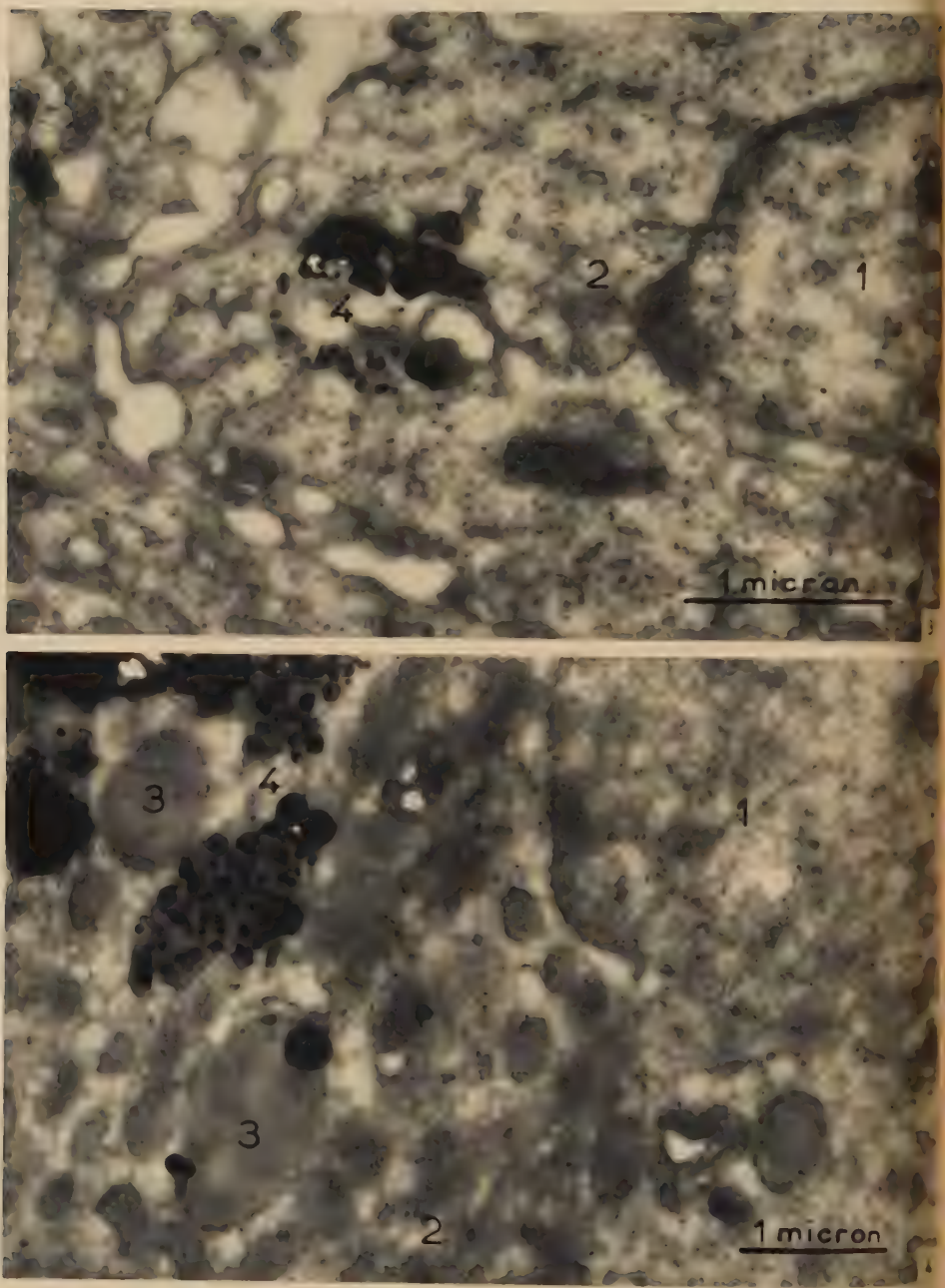
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ment à leur contact. Malgré quelques apparences, il ne semble pas que des ules aient pu pénétrer dans des mitochondries après ouverture de leur enveloppe. le voisinage des amas de silice certaines structures nous ont paru être des ondries altérées. On peut rapprocher ces observations de celles récemment ées [1].

si au contact immédiat des particules de silice une sorte de gangue protéique ince paraît se former et, à une faible distance de celle-ci à son contact se nt des vacuoles à contenu clair, atteignant de 0,1 à 0,5 micron. On peut évoquer ujet la présence fréquente, en microcinématographie en contraste de phase vacuole très claire au contact des particules de quartz, phagocytées par des ytes. Ces enveloppes protéiques et ces vacuoles paraissent pouvoir être con- es comme des réactions pathologiques du cytoplasma au contact des particules ses. On peut envisager aussi que les enveloppes denses sont des protéines ou bris cytoplasmiques phagocytés avec les particules.

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AN EXCHANGE BETWEEN ^{32}P-LABELED PYROPHOSPHATE AND ATP CATALYZED BY AMINO ACIDS IN UNFERTILIZED SEA URCHIN EGGS[1]

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he following abbreviations will be used: ATP adenosine triphosphate; AMP = denylic acid; CoA = coenzyme A; PP = pyrophosphate; P = orthophosphate; A = amino acids; TCA = trichloroacetic acid; Tris = tris(hydroxymethyl)amino- nihane.

LAND [4] has described an amino acid-dependent exchange of ^{32}PP with ATP enzyme system from rat liver. No breakdown of ATP occurs unless hydroxyl- is added. In the presence of hydroxylamine the hydroxamates of the amino

he present investigations have been supported in part by a grant of Consiglio Nazionale icerche to the Institute of Comparative Anatomy of the University of Palermo.

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Lésions pulmonaires, Rat, après 4 jours. Au contact des particules de silice se trouvent à deux vacuoles sans membrane et à droite une vacuole avec ébauche de membrane. Ces es diffèrent des artefacts montrés par la figure 3. Grossissement : 23,500 ×.

Lésions pulmonaires (Rat) 12 jours, après injection de silice. Le cytoplasme entre les les de l'amas principal est très dense. Mitochondries altérées. Gouttelettes de graisse. sement : 16,500 ×.

TABLE I

Amino acid and pyrophosphate requirements for the ^{32}PP–ATP exchange reaction.

Sample Number	Incubation time (minutes)	ATP c.p.m. per μM	% exchange*
	Experiment I		
(1) E + ^{32}PP + AA mix. 60 μM	15	1,002	34
(2) E + ^{32}PP + AA mix. 120 μM	15	1,520	51
(3) E + ^{32}PP	15	930	31
(4) E + ^{32}P + AA mix. 120 μM	15	21	0.1
(5) E + ^{32}P	15	17	0.1
	15	17	0.1
	Experiment II		
(1) E + ^{32}PP + AA mix. 200 μM	0	59	0.8
(2) E + ^{32}PP + AA mix 200 μM	15	2,600	35
(3) E + ^{32}PP + AA mix. 400 μM	15	3,800	51
(4) E − ^{32}PP	15	1,470	20

Conditions for experiment I: ATP 50 μM, ^{32}PP 48 μM (5950 c.p.m. per μM) or ^{32}P 50 μM (18,000 c.p.m. per M); E = mitochondria-free supernatant of egg homogenate 1 ml, final volume 3.4 ml, incubation at 25°C.

Conditions for experiment II: ATP 30 μM, ^{32}PP 32 μM (14,700 c.p.m. per μM); E = mitochondria-free supernatant of egg homogenate 1 ml, final volume 3.5 ml, incubation at 25°C.

AA mix. = equimolecular quantities of the following amino acids: L-leucine, L-isoleucine, L-histidine, L-tryptophane, L-phenylalanine.

$$* \ \% \text{ exchange} = \frac{\text{Specific activity ATP}}{0.5 \times \text{Specific activity PP}} \times 100.$$

acids, AMP and PP appear as end products. This and subsequent work [5] suggest that this reaction might be involved in the activation of amino acids for protein synthesis. It has also been shown that microbial extracts [12] catalyze an amino acid-dependent ^{32}PP–ATP exchange.

We have observed a similar amino acid-dependent exchange of ^{32}PP and ATP in a mitochondria-free supernatant of unfertilized sea urchin egg homogenate.

Eggs of *Sphaerechinus granularis* collected and washed with filtered sea water were homogenized in tris buffer 0.1 M; sucrose 0.5 M, $MgCl_2$ 0.08 M, NaF 0.04 M, pH 7.5, volume eggs/volume buffer = 1/1. The egg homogenate was centrifuged for 1 hr. at 25,000 g at 4°C and the supernatant was used for the experiments. After incubation, as indicated in Table I, the reaction mixture was deproteinized with TCA and the ATP separated from the ^{32}PP by the Norit A method of Crane and Lipmann [1]. The terminal phosphates of ATP were liberated by acid hydrolysis and the inorganic P [3] and the radioactivity were determined. The results are shown in Table I.

We have also observed that there is an increase in the amount of hydroxamic acids formed when amino acids and ATP, or acetate and ATP, are added to the

TABLE II

Hydroxamic acids formation.

Experiment	Sample Number	Glycine 150 μM	Acetate 150 μM	ATP 2.5 μM	CoA*	hydroxamic acid μM
Undialized	1	+		+		1.1
supernatant	2		+	+		1.4
	3			+		0.8
	4	+				0.6
Dyalyzed	1	+		+		0.9
supernatant	2		+	+		0.1
	3			+		0.2
	4	+				0.3
	5	+		+	+	0.8
	6		+	+	+	1.0

Conditions: Undyalized or dyalized particle-free supernatant 1 ml, NH_2OH 150 μM, final volume 3 ml. Incubation 1 hr. at 25°C.
* 2 mg of a preparation from baker's yeast 50 per cent pure.

particle-free supernatant from homogenates of unfertilized sea urchin eggs. If the particle-free supernatant is dialyzed there is a formation of hydroxamine acids from acetate and ATP. Addition of CoA reactivates the formation of hydroxamates from acetate and ATP, while it has no effect on the formation of hydroxamates from amino acid and ATP.

Eggs from *Paracentrotus lividus*, collected and washed with filtered sea water were homogenized in tris buffer 0.1 *M*; sucrose 0.5 *M*, $MgCl_2$ 0.08 *M*, pH 7.5, volume eggs volume buffer = 1/1. After centrifugation in the Spinco preparative ultracentrifuge at 105,000 *g* an aliquot of the supernatant was used as such, while another aliquot was dialyzed against 0.01 *M* tris buffer, 0.04 *M* $MgCl_2$.

After incubation, 2.3 ml of saturated $(NH_4)_2SO_4$ pH 1 and 0.6 ml of $FeCl_3$ were added to the reaction mixture, which was then centrifuged at 20,000 *g* for 10 minutes, the supernatant filtered off and read at 520 mμ. Glycinhydroxamate was used as the reference standard.

The results are reported in Table II.

From the data presented it follows that unfertilized sea urchin eggs have an enzyme system for the activation of the carboxyl group of amino acids. The biochemical and physiological aspects of the problem are being studied.

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A SCANNING INTERFERENCE MICROPHOTOMETER

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The dry mass in biologic material can be determined by interferometry [1,

$$M = \frac{\phi}{\psi} A,$$

where M = dry mass,
ϕ = optical path difference between the material and the embedding medium (o.p.d.), and
$1/\psi$ = proportionality factor.

ϕ can be determined in different ways. In the Stockholm Symposium on Cytochemical Methods with Quantitative Aims, September 1956 [6] a description was given of a scanning interference microphotometer in which the intensity transmitted through the biologic preparation is measured. The intensity is then transformed into optical path difference (o.p.d.). Accordingly, the device can in principle be used with any interference microscope through which the intensity is a fixed function of

The arrangement in the apparatus described may be seen in Fig. 1. The microscope, *I*, projects an image of the specimen onto a photomultiplier, *M*, in front of which a diaphragm, *L*, cuts out a small part of the image corresponding to an area in the preparation of about 0.2 μ^2. The current from the photomultiplier goes to a modified Speedomax recorder [2]. The deflection of the recorder is proportional to the intensity. The preparation is placed on a special stage, *H*, which can be moved with a precision micrometer screw. This screw is driven by a Selsyn motor, *O*. The transmitter is in the recorder and is driven by the recording paper feed motor. Therefore, the movement of the paper and that of the preparation are synchronous. By tilting the mirror, *J*, the measuring line may be moved laterally.

A curve representing a line across the preparation is obtained in the recorder. This intensity curve is transformed in a function transformer into an o.p.d. curve [4, 5], and the integral for the o.p.d. curve is obtained simultaneously. The total dry mass for the measured area of the object is obtained from the integral.

$$M = k \cdot \text{integral},$$

where k = proportionality factor.

This interference microphotometer has been modified to record the o.p.d. directly instead of the intensity. The method has been developed for the Baker Interference Microscope. Plane-polarized light is used in this. Its vibration direction after passing through the microscope is determined by the o.p.d. in the object. In intensity mea-

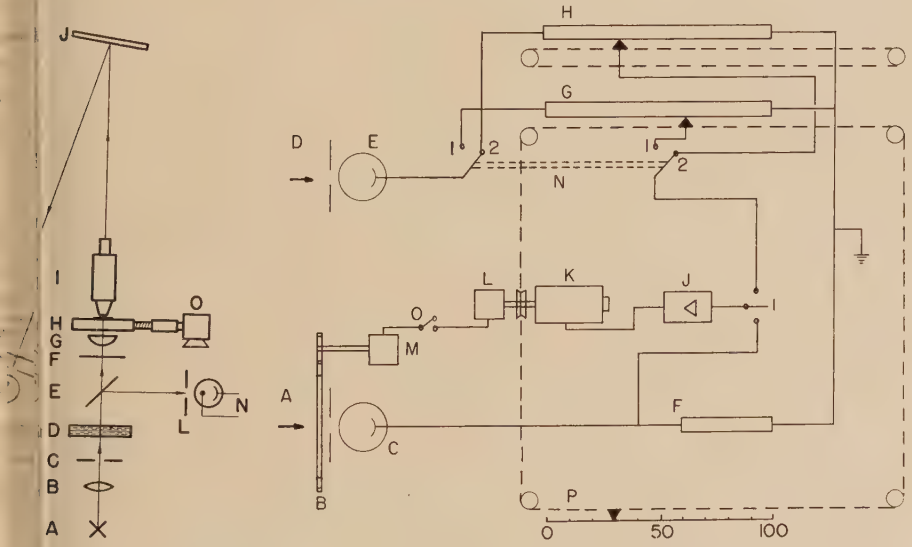
the light also passes through a polarizer (K in Fig. 1), after which the ...y varies as follows:

$$I = I_0 \sin^2 \frac{\phi}{2}.$$

...larizer can also be used for determination of the vibration direction of the ...t. ...r this purpose the polarizer is set to transmit a certain intensity, e.g. mini... ...r different parts of the preparation. The difference between the settings for ...erent points gives the o.p.d. This setting is coupled to the recorder so that ...tion will be directly proportional to the rotation of the polarizer. Accordingly, ...ection of the recorder is proportional to the o.p.d.

...nciple the instrument is constructed as shown in Fig. 1. Fig. 2 shows how ...rizer B (K in Fig. 1) is coupled to the recorder. The apparatus is designed ...t may easily be used for either intensity or o.p.d. measurement.

...urrent from the photomultiplier, C (M in Fig. 1), passes through a fixed ...e F. The current from the photomultiplier, E (N in Fig. 1), passes either ...lancing potentiometer, G, of the recorder or to a manually regulated potentio... ...r I. The amplifier, J, amplifies the impulses coming from the vibrator, I, and ...e balancing motor, K, of the recorder. The rotation of the balancing motor



...(...).—Diagram of the optical part of the scanning interference microphotometer. A Light ...ce; Lens; C Diaphragm, D Filter; E Light splitter; F Polarizer; G Condensor; H Stage; ...crope; J Mirror; K Polarizer; L Diaphragm; M and N Photomultipliers.

2. (*...ght.*).—Diagram of the electrical part of the scanning interference microphotometer. ...ea...ing light beam; B Polarizer; C Measuring photomultiplier; D Compensating light beam; ...m...sating photomultiplier; F Resistor; G and H Potentiometers; I Converter; J Amplifier; ...ala...ng motor; L Selsyn transmitter; M Selsyn receiver; N and O Switches; P Indicating ... ...e recorder.

is transmitted by Selsyn motors, L and M, to the polarizer. The transmitter of the Selsyn motors is attached to the balancing motor. In intensity measurement the selector switch, N, is in position 1, coupling in the balancing potentiometer of the recorder. The switch, O, is open so the rotation of the balancing motor is not transmitted to the polarizer. The recorder's deflection, P, is thus proportional to the intensity on the photocell, C. In direct o.p.d. measurement the selector switch is in position 2, coupling in potentiometer H.

The measuring procedure is as follows: The instrument is first set up for intensity measurement (switch N in position 1 and switch O open). The light intensity to the photocell C for the o.p.d. measurement is determined. This should be the mean between maximum and minimum values for different settings of the polarizer as the sensitivity is greatest there. That is to say $dI/d\phi$ is at maximum. $d\phi$ = the change in o.p.d.: dI = the corresponding change in the intensity on C when the polarizer remains in the same position. With the polarizer in a corresponding position and switch N in position 2 and with the balancing motor serving as a null instrument, potentiometer H is manually adjusted so that its slide wire gives the vibrator, K, the same potential as resistance F. Now, when switch O is closed, the balancing motor of the recorder automatically controls the position of the polarizer so that the photocell receives unchanging light intensity with varying o.p.d. The angle through which the polarizer is rotated for a certain change in o.p.d. is thus transmitted to the recorder pen and the deflection will be directly proportional to the o.p.d.

The linearity was tested by measurement on a thin plane-convex lens along a line passing through the centre of the lens. A shearing objective was used for this. The o.p.d. in a shearing objective between the two images of the lens varies approximately linearly along a straight line. Therefore, its record should be a straight line. The deviation from linearity was less than ± 0.5 per cent. This linearity test is a check on both the deflection of the recorder and the movement of the specimen.

The scale factor of the recorder can be easily varied by changing the gearing between the balancing motor and the polarizer.

The total dry mass for a scanned area in the specimen can be obtained by planimetry of the curves:

$$M = kA,$$

where M = total dry mass,
k = proportionality factor, and
A = area measured by planimetry.

The curves can be integrated in an integrator [4, 5] manually or automatically by connecting the integrator directly to the recorder. The integral is then obtained at the same time the measurement is carried out on the preparation.

The reproducibility in direct o.p.d. measurement was tested by repeated area scanning of the same ascites tumor cell. The results are presented in Table I. The maximum deviation from the mean of ten measurements is about 3 per cent, and the probable error in the individual measurement is less than ± 2 per cent.

The instrument has proved easy and rapid to work with on different kinds of material. It has been especially useful in measurements of large series of ascites tumor cells.

TABLE I

Repeated area scanning of ascites tumor cell.

Measurement No.	$\int \phi(A)\, dA$ cm³
1	29.9×10^{-12}
2	29.8
3	29.2
4	30.4
5	29.5
6	28.8
7	28.8
8	29.6
9	28.9
10	29.2
Mean value	29.4

Summary.—An instrument for direct measurement of the optical path difference and in biologic preparations is described. The instrument includes a Baker terference Microscope. A small area (0.2 μ^2) of the preparation is projected onto photomultiplier. The analyzer of the microscope is located in front of the photo- multiplier. This analyzer is driven by a servo system so that the intensity on the photomultiplier remains constant. The deflection of the recorder pen coupled to the servo system is proportional to the rotation of the analyzer and thus also to the and. The object is scanned along parallel lines by moving the preparation, and photo. curves are recorded. The area under the curves (the integral) is proportional the dry mass of the scanned area.

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OBSERVATIONS ON THE ANISOTROPIC BAND OF CROSS-STRIATED MUSCLE

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In view of the recent work on the localization of the contractile proteins within the myofibrils, the anisotropic band (A band) of muscle has become a central concern of investigators. The results obtained by Hasselbach [4] and by Hanson and Huxley [2, 5] have led them to conclude that myosin is localized in the A band of the myofibril in the form of filaments approximately 110 Å in diameter extending from the isotropic band (I band) to the lighter central zone of the A band, the H band, and perhaps, also residing in the 140 Å filaments of the H band [5]. Other workers [11, 12] suggest that this conclusion is premature since they have found protein material, in addition to myosin, in the extracts obtained when the A band of isolated myofibrils is removed by treatment with myosin solvents [2, 4, 5].

Whether myosin will be proven localized in the A band of muscle or will be found to exist continuously throughout the filaments of the sarcomere, it is quite obvious that the A band must play a role in the contraction of striated muscle. Observations on the behavior of the A band in stretch and contraction evidence, at present, some disagreement. Some workers [1, 9, 10] find that during stretch both the A and the I bands are elongated, whereas others [5, 6] find that there is no significant increase in the length of the A band in stretch. During isometric contraction the former [9] find that the A band shortens while the I band elongates, and the latter investigators [5, 6] find that the band lengths do not change. The results described here indicate that increased length of the sarcomere, as a result of stretch, in all probability, depends primarily upon the increased length of the I band. There may be a slight increase in the A band although the increase is below the resolving power of the microscope. On the other hand, as has been indicated by previous workers [5, 10], changes within the A band, in stretched preparations, can be observed. It has been suggested [7, 8, 9] that these changes may be involved in the formation of the contraction bands.

Methods.—Psoas fiber bundles were prepared from freshly killed (decapitated) rabbits in the following manner: rest length bundles, by tying both ends of the fibers to a splint before cutting from the rabbit; equilibrium length bundles, by tying one end of the fibers to a splint, cutting the other end allowing the muscle to shorten freely, and then tying the free end to the splint; stretched fibers, by tying one end to a splint, cutting and stretching the muscle at the other end to about 140 per cent rest length and then tying that end to the splint. All fiber bundles tied to splints were then placed in 50 per cent aqueous glycerol at 0°C and stored at that temperature for 24 hours. They were then transferred, still in 50 per cent glycerol, to a deep freeze at −20°C for a period of 10 or more days. It was assumed that fibers so treated

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Table I

sarcomere length (1)	Observed I band + Z, μ (2)	Observed H, M, & E band, μ (3)	Observed 2 outer dense A, μ (4)	(A band) sarcomere, μ 3+4 (5)	2+5	A:I
3.3	1.80	0.97	0.55	1.52	3.32	0.8
3.2	1.69	0.85	0.57	1.42	3.11	0.8
3.1	1.70	0.83	0.48	1.31	3.01	0.8
2.9	1.52	0.63	0.84	1.47	2.99	1.0
2.8	1.35	0.58	0.86	1.44	2.79	1.1
2.7	1.28	0.50	0.90	1.40	2.68	1.1
2.6	1.24	0.51	0.89	1.40	2.64	1.1
2.5	1.10	0.41	0.92	1.33	2.43	1.2
2.4	1.06	0.30	1.06	1.36	2.42	1.3
2.3	0.95	0.31	1.06	1.37	2.32	1.4

re the relaxed condition; in any case contraction could be elicited by treatment h ATP in a KCl-$MgCl_2$ solution. It is, however, possible that they were isometri-ly extracted.

For phase contrast observation, thin strips of psoas, about 2 or 3 fibers thick, were sec from a fiber bundle in 5 per cent glycerol. They were placed on slides, covered i e mined under oil immersion at 1125 X with a Leitz Dialux microscope. oto aphs were taken and enlarged to a final magnification of 3600 X for the actual as ments.

Res s and Discussion.—Measurements were taken of the whole sarcomere, the and ncluding the Z band of one sarcomere, the dense outer edges of the A band, i th inner, light portion of the A band consisting of the H, M, and E[10] bands. e re lts are summarized in Table I; each figure represents an average of at least nea rements. The sarcomere length (column 1) has been rounded off to the first im place for ease of grouping the other measurements. The accuracy of actual asu ment for all figures is ± 0.05 μ although it must be remembered that the olu power of the microscope is somewhat less than this.

t i eadily apparent that in stretch from a sarcomere length of 2.3 μ to one of μ .5 per cent increase in length from an approximate equilibrium) that the I d s one Z almost doubles in length (1.0 μ to 1.8 μ). The A band (column 5) ws slight increase (1.4 μ to 1.5 μ) over this range, although it must not be con-re ignificant in view of the resolving power of the light microscope. The ratio the :I band does not reflect the almost constant ratio of 1:0.7 obtained by Isc nd Knappeis in living muscle at all degrees of stretch nor does it reflect lat ratios obtained by these workers in fixed muscle; 1:6 at low stretch and .S high stretch. In electron micrographs taken of blended, washed, air dried un ained myofibrils, the A band (not including the M line) increased slightly, μ 1.43 μ, while the major increase in the sarcomere length from 1.7 μ to 2.7 μ ap rently due to the I band's increased length, 0.34 μ to 1.01 μ (not including Z l nd).

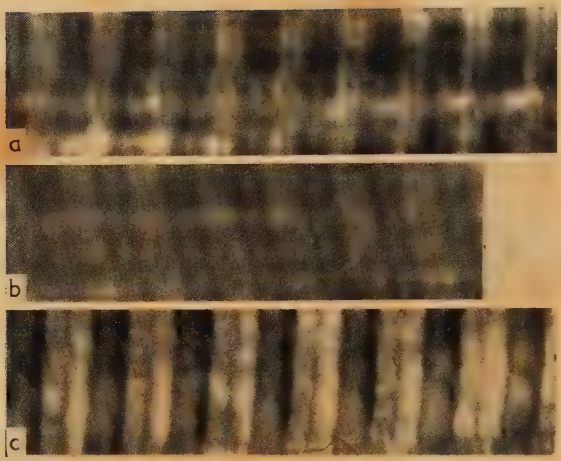

Fig. 1.—(*a*) Relaxed muscle at eq librium length. Sarcomere equals 2.4 (*b*) Relaxed muscle at rest length. S comere equals 2.6 μ. (*c*) Relaxed mus at length approximately 120 per c of rest length. Sarcomere equals 3.1

Within the A band large changes can be noted. The H zone, composed of t H, M and E bands, seems to increase at the expense of the dense regions of t A band: that is the dense zone becomes thinner as the muscle is stretched from equilibrium position (Table I and Fig. 1 *a*, *b*, *c*). The H zone is virtually impossib to see in 0.85 per cent NaCl or in formol fixed fibers. The light zone can be see however, in preparations of psoas myofibrils which have been washed several tim before observing either in weak salt solution under phase contrast or in dried pr parations under the electron microscope.

When one considers what a small part (less than 20 per cent) of the sarcomere th dense regions of the A band occupy at a sarcomere length of 3.3 μ which is wi within physiological stretch limits, it is hard to imagine all the myosin of muscle this region [2, 3]. It would appear, from a morphological viewpoint, far more logic to assume that myosin and actin are distributed from Z to M, through the H zon with some other material [11, 12, 7, 8] "sitting" on or with the actomyosin filamen in the dense A band. Support for extra material in the A is afforded by the excelle electron microscope studies by Hanson and Huxley [2] and others which show different filamentous structure in the A and I regions.

Summary.—During passive stretch of muscle both the I and the centrally locate H, M, E regions increase while the dense outer region of the A band decreases in length. Whether the total A band remains constant in length or increases ve slightly in the range studied cannot be categorically stated in view of the sm changes noted which are below the resolving power of the microscope.

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AC[illegible]-SOLUBLE NUCLEOTIDES IN THE EARLY DEVELOPMENT OF *PSAMMECHINUS MILIARIS*

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[illegible]a[illegible]been shown previously [3–8] that the early stages of sea urchin development [illegible]ch[illegible]acterized by a rapidly intensified metabolism of nucleotides and structural [illegible]te[illegible]. The most rapid increase is concentrated to the early blastula stage, i.e. to [illegible]p[illegible]od of primary determination [10]. During this early period the energy pro-[illegible]tic[illegible] of the embryo is not very extensive [11]. The increase of the mitochondrial [illegible]ter[illegible]loes not take place until slightly later, during the gastrula stage [1, 2].

'[illegible]h[illegible]ollowing experiments show, on a semi-quantitative basis, that there are [illegible]sic[illegible]able amounts of nucleoside pyrophosphates accumulated in the unfertilized [illegible]. [illegible]uring the early development these compounds rapidly diminish. A more [illegible]er[illegible]adecrease in the amounts of acid-soluble purine and pyrimidine derivatives [illegible]:in[illegible]ced in the late stages of development.

[illegible]xperimental.—Dilute suspensions of eggs from the sea urchin *Psammechinus [illegible]ar[illegible]*(Gmelin) were fertilized and allowed to develop at 18–19°C in motor-driven, [illegible]cin[illegible]glass-trays. Before fertilization, and at different stages of development [illegible]ive[illegible]nt samples of eggs or embryos were withdrawn from the culture. After [illegible]tri[illegible]gation at low temperature the material was extracted at 0°C with perchloric [illegible]i b[illegible]he method of Hurlbert *et al.* [9]. After neutralization with KOH and centri-[illegible]ati[illegible] the extracts were preserved at −20°C until all the samples had been obtained [illegible]n[illegible]t culture. The extracts were then placed on columns of the anion exchanger [illegible]ve[illegible] (formate form). Because of the limited amounts of embryonic material at [illegible]os[illegible] the columns were made very narrow (0.13 cm^2 × 21 cm). Column tubes of [illegible]eti[illegible]he same dimension had been selected, and all columns were packed at the [illegible]e[illegible]e and under identical conditions.

[illegible]he[illegible]romatograms were eluted with formic acid and ammonium formate of grad-[illegible]y i[illegible]reasing concentrations [9]. The eluate was automatically collected in samples [illegible]pp[illegible]ximately 1.5 ml volume. The optical density of the samples was measured at [illegible]m[illegible], Absorption spectra were determined in samples with increased readings. One [illegible].da[illegible] column with known compounds, and seven columns with extracts from eggs

and embryos at different stages of development were eluted synchronously, supplied with eluent from the same mixer. Two experimental series of this kind were accomplished in the spawning season.

Because of the limited amounts of material available only the main fractions could be further utilized for proper characterization. These fractions were lyophilized and analyzed by rechromatography by an ammonium formate system [9]. The fractions obtained from these chromatograms were treated with N HCl at 100°C for 60 minutes.

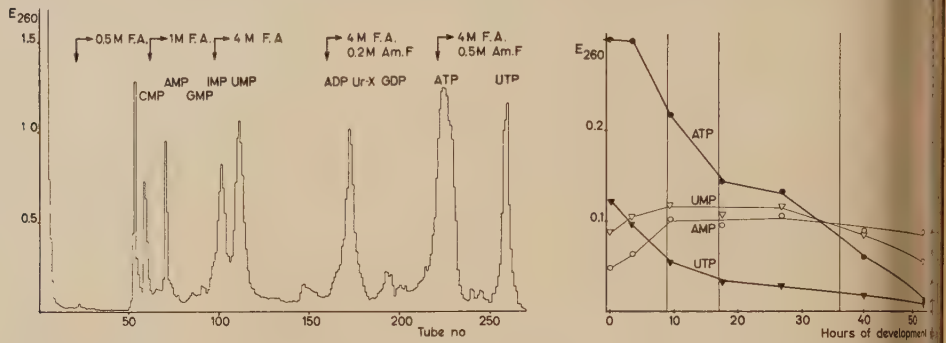

Fig. 1 (*left*). Formic acid chromatogram of the acid-soluble fraction of unfertilized *Psammechinus* eggs (approximately 10^6 eggs). *Column:* Dowex-1 formate, 0.13 cm² × 21 cm). *Mixer:* 100 ml, common to 8 columns. *Reservoir:* water at the beginning, changed as indicated. *Rate of elution:* 1.4–1.8 ml per 30 minutes. The E_{260} of the 30 minutes samples was determined after dilution to 2.5 ml.

Fig. 2 (*right*). The fractions of ATP, UTP, AMP and UMP at different stages of development. E_{260} of the total fractions, recalculated to a standard volume of 100 ml. Vertical lines indicate hatching, start of invagination and appearance of arms.

Under these conditions purine nucleotides are hydrolyzed, but not pyrimidine nucleotides. The reaction products obtained by the HCl-treatment were identified by chromatography [4] on Dowex 50 columns (acetic acid and HCl system). The identity of the purines was finally checked by deamination with $NaNO_2 + H_2SO_4$ [12] and rechromatography on Dowex 50 [4]. The ultraviolet absorption spectrum of the fractions was determined in 0.1 N HCl and 0.1 N NaOH. The identifications indicated in Fig. 1 are based on the analytical data in combination with the elution diagrams of Hurlbert *et al.* [9].

Results.—The chromatogram of a perchloric acid extract from unfertilized eggs is illustrated by Fig. 1. The chromatogram was dominated by the large peaks in the pyrophosphate region. ATP occurred in large amounts while the AMP-fraction was rather small. UTP also was present in relatively great quantity cf. [4, 8]. During the course of development the character of the chromatograms gradually changed (Fig. 2). Especially during the blastula stage the content of nucleoside pyrophosphates rapidly decreased, while instead the amounts of the corresponding monophosphates increased. The utilization of the store of nucleoside pyrophosphates may be connected with the increased metabolism of nucleic acids and structural proteins during the determination period and with the active ciliary movement of the swimming embryos. During the gastrula stage, when the enzymatic systems associated

th itochondria increase [1], the consumption of nucleoside pyrophosphates mp arily ceased in the material investigated.

in e plutei a successive decrease of all nucleotides, with the possible exception A , was observed. The fraction of nucleotide bases also became considerably iu . The decrease was plausibly correlated with the condition of starvation which ad ly sets in during the late stages of development in pure cultures.

Ch cteristic changes occurred also in the minor nucleotide fractions during devel- me . However, a detailed analysis of these fractions had to be postponed because th imitations of the *Psammechinus* material.

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ODUCTION OF MITOTIC ABNORMALITIES BY ETHYLENE-DIAMINETETRAACETIC ACID[1]

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AN chemical and physical agents can disrupt the normal course of mitosis in ant nd animal cells; and numerous attempts have been made to identify the uc al elements and metabolic pathways involved (see [6, 7] for summary of era re). Experiments employing the chelating agent ethylenediaminetetraacetic d DTA) have focussed attention recently on the possible role of the divalent tio Ca and Mg in maintaining chromosomal integrity [3 5, 12–14, 18, 19]. Our din have demonstrated, however, that the effects of EDTA are probably due a neral interference with the ionic environment of the cell rather than to the eci removal of cations from the chromosomal fabric [8–10]. Since ribonucleo- ote appeared to be involved both in the EDTA-induced dispersal of chromatin 1 and the increased degree of crossing over noted in *Drosophila* [8, 9], and

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since ribonuclease had been shown to produce mitotic abnormalities in growing root-tip cells [6, 7], it seemed advisable to compare the effects of EDTA on the cells of growing roots with those previously obtained with ribonuclease. This comparison has confirmed the hypothesis that any agent which interferes with cellular metabolism can induce chromosomal abnormalities.

Materials and methods.—Growing roots of onion (*Allium cepa* L) were immersed in aerated solutions of EDTA (and the other reagents tested) for periods of time ranging from 2 to 24 hours at room temperature (ca. 22°C). The roots were excised at selected time intervals, fixed in acetic acid-alcohol, washed with 70 per cent alcohol, and squashed in aceto-orcein for microscopic examination. All solutions were made from analytical reagent grade chemicals, using tap water (containing ca 1.5 p.p.m. Mg and 6 p.p.m. Ca) as the solvent; the desired pH's were obtained by titrating 0.2 *M* solutions with appropriate concentrations of NaOH, and diluting the resulting stocks to the concentration desired.

Results.—The nature and frequency of the mitotic abnormalities produced by EDTA were found to be related to concentration of the chelating agent and to the duration of treatment; they were practically independent of pH in the range between 6 and 7. Concentrations of EDTA greater than 0.002 *M* were in general toxic, producing in less than 6 hours marked pycnosis, particularly in the peripheral regions of the roots. In these cases EDTA appeared to arrest all mitotic processes, after which structural deformation occurred.

Concentrations of EDTA between 0.0004 and 0.002 *M*, when used for periods ranging from 2 to 10 hours, produced all the types of chromosomal aberrations and mitotic abnormalities effected previously by ribonuclease [6, 7]. Photographs of such aberrations have been published elsewhere [7, 9]. The first noticeable effect was enlargement of the nucleoli in interphase and prophase nuclei. Subsequently the meristematic cells revealed anaphase and telophase bridges (attributable to induced stickiness of chromatin), excessive contraction of metaphase chromosomes, polyploid chromosome complexes, multipolar spindles, and multinucleate complexes. Chromosome fragments were observed only rarely, which suggests that solutions of EDTA do not effect direct breakage of chromosomes in cells of the root-tip meristem (cf. [6, 7]).

In order to determine whether or not Ca or Mg ions were involved in the reaction systems responsible for the production of these abnormalities, growing onion roots were treated with 0.002 *M* $CaCl_2$ or $MgCl_2$, either alone or in combination with equimolar concentrations of EDTA. Mitotic abnormalities, similar to those noted with EDTA, were produced by either $CaCl_2$ or $MgCl_2$ [9], the former being slightly more efficient than the latter. Under the same experimental conditions, the EDTA chelates of Ca and Mg were found to be less effective than either component alone.

Treatment of growing onion roots with ribonuclease under certain experimental conditions results in the excretion of deoxyribonuclease [1]. In preliminary experiments with EDTA, no such release of deoxyribonuclease or of deoxyribonucleic acid was found. There was excreted, however, an as yet unidentified substance, which reacts with Dische's diphenylamine reagent [2] to produce a green compound with an absorption maximum at 650 mμ and a minimum at 550 mμ.

Discussion.—Hyde [4, 5] has reported that treatment of growing root tips of onion or broad bean with 0.01 *M* EDTA for 30 to 45 minutes slowed division for

veral days but did not permanently affect chromosome structure. Treatment for ger periods of time (ca 3 hours) usually had lethal effects, as manifested by the pression of anaphase movement and marked swelling of late prophase and taphase chromosomes. This swelling continued until discrete chromosomes could longer be distinguished. Ca appeared to be the critical ion chelated in Hyde's periments, since roots treated with 1:1 molar ratios of $CaCl_2$ and EDTA continued divide normally whereas treatment with similar solutions of $MgCl_2$ and EDTA duced results resembling those obtained with EDTA alone. The factors responsible the discrepancies between his results and ours are not at present clear.

Numerous investigators have shown that exposure of various plant tissues to lutions of neutral salts resulted in the formation of chromosomal abnormalities e [11, 15–17, 20] for representative literature). Their results have in general been nfirmed in the studies reported here with $CaCl_2$ and $MgCl_2$. If the primary mode action of EDTA in producing chromosomal abnormalities involved removal of alent cations that normally bind together the macromolecular constituents of chromosomes, it is difficult to understand why addition of divalent cations ould have a similar effect. It seems more consistent with the findings reported re and with those presented in our earlier reports [8–10] to attribute the structural tability effected by EDTA to alterations in the general metabolism of the cell [3, 12, 13, 17].

Summary.—Treatment of growing roots of onion with solutions of EDTA between 0.004 and 0.002 *M* was found to modify the form and distribution of the chromosomes of the meristematic cells. Similar results were obtained with 0.002 *M* $CaCl_2$ or $MgCl_2$. The effects of EDTA are attributed to alterations in the general metabolism of the cell, rather than to direct breakage of chromosomes through chelation of divalent cations serving as bridges between macromolecular complexes of protein and nucleic acids.

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CORTICAL INHIBITIONS AND POLYSPERMY IN ANTISERUM-TREATED SEA URCHIN EGGS[1]

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When unfertilized sea urchin eggs are exposed to high concentrations of homologous anti-rabbit sera, a number of response reactions (e.g. precipitation of the jelly layer, parthenogenetic activation) can be observed under the microscope [4, 5]. Treatment of the eggs under milder conditions does not bring about these visible alterations but appreciably lowers the fertilizability of the eggs. In a previous paper [6] it was shown that the reduction of fertilizability was due to the action of species- and egg-specific antibodies which apparently did not affect the sperm. There the main stress was layed on a description of the action of antiserum on the *rate of fertilization*. However, it was also mentioned that antiserum treatment of the eggs previous to fertilization led to considerable disturbances of ensuing cell division and development. The purpose of this paper is to describe these disturbances. The results of the analytical methods applied in order to resolve the "cytotoxic" action of antiserum on the eggs into some of its most distinct components will be presented later.

Material and methods.—The experiments were performed at the Stazione Zoologica of Naples, using mainly the eggs of the sea urchin *Paracentrotus lividus*. Rabbit antisera against unfertilized eggs, sperm and jelly substance were produced and brought to isotonicity with sea water as previously described [5]. All sera were heated to 56°C for 30 minutes before use. Further details concerning the sera will be given in the text below.

The eggs were collected according to [3] and were treated with antiserum in covered dishes at constant temperature. 0.5 ml of the concentrated egg suspension, adjusted to contain 100,000–200,000 eggs (determined accurately by counting egg numbers in diluted aliquot samples) from 1–3 females were mixed with 1 ml rabbit serum. The serum was a mixture of varying volumes of normal rabbit serum and antiserum, thus giving constant amounts of serum proteins but varying antiserum concentration (v/v) in all experiments. Antiserum concentration was expressed as the egg/antiserum ratio, obtained by dividing the actual number of eggs in the experiment by the volume of antiserum used (ml). The time of the treatment was in all cases 40 minutes, which was found in pilot experiments to be sufficient to give optimal effects over the whole range of antiserum concentrations. During the treatment the bowls were shaken occasionally.

After the treatment the eggs were transferred to beakers and washed by decantation three times with sea water. Microscopically undamaged samples were then fertilized, using 6×105 sperm/ml final volume at sperm/egg ratios of ~ 300. The incidence of polyspermy was established by counting of 250 eggs in each experiment before the occurrence of the first mitotic division [1]. Eggs containing supernumerary

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rm in the cytoplasm were considered polyspermic. The development of the eggs s followed up to about 50 hours after fertilization with observation of aliquot nples (250 eggs) at the blastula and pluteus stages.

ontrol experiments were always run with eggs in sea water and eggs pretreated h normal rabbit serum. Samples showing incomplete fertilization, polyspermy or elopmental disturbances in the controls were discarded. The incidence of poly- rmy in the controls was rare under the experimental conditions used.

Results.—Activation of unfertilized eggs by anti-egg serum applied in high con- tration is generally accompanied by microscopically visible inhibition phenomena heir cortical zones [4, 5]. Similar disturbances are found in eggs fertilized after treatment with antiserum under milder conditions. The affected eggs are in h cases characterized by a locally inhibited breakdown of the cortical granules, inhibition of the formation of the fertilization membrane (membrane blisters), hological development of the hyaline layer, and more or less pronounced inhibition nuclear and cytoplasmatic cleavage.

n Table I the results of a number of experiments are given. All experiments were formed independently on eggs from different females, using the same anti-egg m throughout. In Table I the experiments have been arranged in the order of reasing intensity of the antiserum pretreatment (column 1). Sea water and normal bit serum controls, which were negative, are not included.

olumn 2 gives the total number of visibly affected eggs in each experiment. These res include both eggs rendered unfertilizable by the pretreatment and eggs with ical inhibitions of the type described above. Eggs developing to microscopically mal plutei have been classified as unaffected. This does not mean that all of these s were entirely unchanged: compared to the controls a slight and apparently siologically harmless retardation of cleavage could still be found in some of them.

s can be seen from Table I, the percentage of the affected eggs decreases rather ly with decreasing antiserum concentration. As has been established in a great mber of experiments, a rather good linear relationship holds between the total mber of affected eggs and the egg/antiserum ratio. For the antiserum used in this

TABLE I

Act of homologous anti-egg serum in varying concentrations on fertilization of the eggs of Paracentrotus lividus.

The percentage figures are based on counts of 2×250 eggs in each experiment. For further explanations see text.

Egg/antiserum ratio	Affected eggs (%)	Fertilized eggs (%)	Polyspermic eggs (%)
1.2×10^5	98	20	?
1.3×10^5	97	83	4
1.5×10^5	85	88	?
1.8×10^5	95	93	31
2.4×10^5	70	90	26
2.7×10^5	67	98	29
3.6×10^5	40	97	14

series, this relationship ranges from almost 100 p.c. affected eggs at ratios of approximately 1.25×10^5 to less than 5 p.c. at ratios of 5.25×10^5 or higher.

The figures in column 2 are only influenced to a slight degree by the proportion of eggs rendered unfertilizable by the antiserum pretreatment (column 3). At the lowest egg/antiserum ratio given in Table I, 80 per cent of the eggs remained unfertilized. The response curve for "fertilizability" as a function of antiserum concentration had a marked point of inflection: in 10 experiments with the antiserum under consideration applied at egg/antiserum ratios of 1.0×10^5–1.2×10^5 80–100 p.c. of the eggs remained unfertilized; the mean number of unfertilized eggs in 9 experiments at egg/antiserum ratios of 1.3×10^5 2.0×10^5 was 11.0 ± 2.2 p.c.; and in 10 experiments at egg/antiserum ratios of 2.1×10^5–2.6×10^5 it was 6.5 ± 1.9 p.c.

From the type of the cortical response in the antiserum pretreated eggs it also seemed reasonable to expect an increased incidence of polyspermic fertilization. The incidence of polyspermy is shown in column 4 of Table I, which gives the number of polyspermic eggs as percentage of fertilized eggs in each experiment. The figures actually represent minimum numbers as a certain "roughness" of the egg surface after antiserum pretreatment sometimes rendered observation difficult. This concerns especially the first three records where practically no polyspermy could be observed. However, with regard to the low fertilizability of the eggs at low egg/antiserum ratios it seemed likely that polyspermy was actually inhibited in these three experiments.

The *anti-egg serum* used in this series of experiments was the serum pool from 5 rabbits, each injected with the same lyophilized egg homogenate (method of presensitization, 100 mg egg nitrogen/rabbit, cf. [5]). Tests with two other homologous anti-egg sera, obtained from 5 rabbits each and using different egg materials and different injection schedules, all gave inhibition phenomena of the same kind. However, it must be emphasized that the titers for the different antiserum activities (50 per cent cortical damage and inflection point for "unfertilizability") differed appreciably and independently for each antiserum used. Details will be given later.

Of special interest were the tests made with *two anti-jelly sera* (*Paracentrotus*), each serum being prepared from 3 rabbits. In the first, 450 mg jelly substance was injected per rabbit. The jelly was isolated at pH 5.2, purified by repeated precipitation with absolute ethanol [7] and lyophilized after dialysis. In the second, 125 mg were injected per rabbit. The jelly was isolated in the same way but lyophilized after dialysis and pervaporation without further purification. Both preparations were injected as emulsions with adjuvant (cf. [5]). While the anti-egg sera referred to above were practically devoid of jelly precipitating activity when tested in high concentrations on unfertilized eggs [4, 5], both anti-jelly sera were strong jelly precipitants with approximately the same end-point for 50 per cent visible response, at egg antiserum ratios of 5×10^3–1×10^2. Pretreatment of the eggs with the antiserum against the *purified* jelly preparation caused no inhibition whatsoever upon subsequent fertilization with egg/antiserum ratios ranging from 5×10^4–5×10^5 in 7 experiments. Simultaneous tests with anti-egg serum gave strong responses. The serum against the *crude* jelly preparation gave no response in 3 of the 7 experiments. In 4 experiments a *weak inhibition of cytoplasmatic cleavage* could be noticed with no inhibition of nuclear cleavage.

One antiserum against lyophilized egg homogenate of *Arbacia lixula* and one against *Psammechinus microtuberculatus* (5 rabbits each) exhibited a very low

i biting activity on the *Paracentrotus* eggs. Both antisera had a high inhibiting acity when tested on the homologous eggs.

ll *normal rabbit sera* tested (2 serum pools from 5 rabbits and 3 individual sera) wed only the general improving action on fertilization previously recorded [6]. only, strongly sperm agglutinating *anti-sperm serum* (*Paracentrotus*) tested (5 bits, each injected with 80 mg nitrogen of a lyophilized sperm homogenate) was pletely inactive with respect to both cortical damage and inhibition of fertiliza- rate.

Discussion.—The inhibiting action of antiserum described in this note is due to presence of antibodies directed against macromolecular, species-specific structures he egg. There is good evidence for the assumption that the inhibiting reactions ribed here, as well as the parthenogenetic activation described earlier [4, 5] are narely due to a number of distinct serological reactions occurring in the surface rs of the egg. An extensive discussion of the problems involved will be postponed l more evidence has been presented.

t is noteworthy that a decrease of antiserum concentration does not bring about adual transition in the test from total inhibition of fertilization to more or less nounced inhibition of the visible cortical reactions. Instead, there seems to exist a er limited and distinct range of concentration for each antiserum, within which response of most of the eggs changes from total unsusceptibility to the sperma- a to fertilizability. The mechanism of this block is not yet understood. The rtilizable eggs look normal in the microscope and can still be activated partheno- etically.

he linear relationship between the concentration of the antiserum and the number ortical inhibited eggs implies a rather direct correlation between the antigen- body reactions and this particular microscopic response. Whatever its mechanism, response in its microscopic features resembles that exerted by a number of stances known to induce polyspermy [2] and it thus was not surprizing to find ificant figures for polyspermy also in the present case. Polyspermic fertilization t at least in part be responsible for the pathological development observed in ted eggs.

nhibition of nuclear division occurring in the eggs after pretreatment with anti- m in higher concentration is rather typical for "partially fertilized" eggs [1] and be causally related to the block of the cortical reactions. This may also be the with the inhibition of cytoplasmatic cleavage though there are reasons to suspect t least partially independent mechanism of antiserum action.

s previously shown, the jelly layers of the *Paracentrotus* eggs as well as those of r Mediterranean sea urchins are highly antigenic [4, 5]. From the experiments anti-jelly sera referred to above it seems quite obvious that the antibodies "precipitating" the jelly layer are not capable of evoking any noticeable inhibiting tions of the type described in this paper.

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CORRÉLATION ENTRE LA MICRORADIOGRAPHIE ET L'IMAGE EN LUMIÈRE POLARISÉE DE L'OS SECONDAIRE[1]

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L'aspect de l'os secondaire en lumière polarisée est bien connu : entre nicols croisés les lamelles osseuses sont alternativement claires et sombres. Récemment, les progrès de la technique microradiographique ont permis de reconnaître, dans le même tissu, la succession de stries qui se distinguent les unes des autres par leur pouvoir d'absorption des rayons X [2, 3, 7, 8]. Au surplus, dans nos recherches antérieures, nous avons remarqué que les stratifications observées sur la microradiographie semblent répondre en nombre à celles de l'image en lumière polarisée. Il nous était toutefois impossible de décider si les lamelles les plus opaques aux rayons X étaient éclairées ou non en lumière polarisée. La présente note reprend cette question.

Technique.—Des fragments osseux sont prélevés chez des sujets humains adultes au cours d'intervention chirurgicale ou à l'autopsie. Ils sont fixés dans de l'éthanol à 96° et enrobés dans du méthacrylate de méthyle suivant la méthode décrite précédemment [8]. Grâce au microtome Jung (modèle K), on débite des coupes transversales et des coupes longitudinales épaisses de 10 microns.

Ces coupes sont microradiographiées à l'aide de l'appareil Philips réglé à 2,5 kV et 2,5 mA. On emploie le film spectroscopique 649-GH d'Eastman Kodak, exposé pendant 10 minutes à la distance de 14 mm. Après développement et fixation, le film est lavé, séché à l'abri des poussières et monté au baume de Canada.

Les coupes elles-mêmes sont à leur tour montées dans du chlorure de sodium à 10 % et sont photographiées entre nicols croisés à un grossissement rigoureusement identique à celui qui a été choisi pour reproduire le cliché microradiographique.

Quelques coupes ont été décalcifiées pendant douze heures dans une solution de séquestrène Na_2 à 5 % avant d'être examinées en lumière polarisée.

Observations.—La figure 1 représente un segment d'ostéone en coupe transversale. Sa moitié gauche est réservée au négatif microradiographique, sa moitié droite à l'image en lumière polarisée. La structure lamellaire est évidente sur les deux clichés. Si l'on cherche à faire correspondre les lamelles, on constate que les bandes les plus opaques aux rayons X sont, en grande partie, éclairées en lumière polarisée.

La figure 2 offre à considérer en haut la microradiographie et en bas l'image en lumière polarisée d'une coupe longitudinale d'un ostéone. On voit, comme sur la coupe transversale, qu'une lamelle donnée est blanche ou noire à la fois sur les deux clichés.

Une correspondance analogue ressort de l'examen de la figure 3 fournie par une travée d'os spongieux.

Dans les illustrations que nous présentons, le grossissement employé fait inévitablement apparaître le grain de la radiographie, mais il rend plus certaine l'identification des lamelles. D'ailleurs, au laboratoire, cette identification est aisément confirmée

[1] Ce travail a été effectué en partie grâce à une subvention du Fonds National Belge de la Recherche Scientifique.

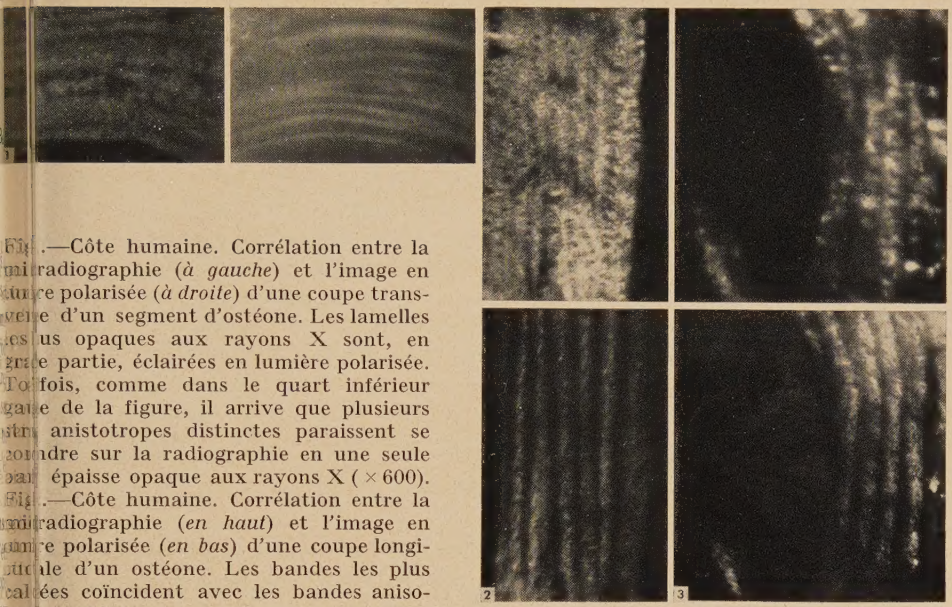

Fig. 1.—Côte humaine. Corrélation entre la microradiographie (*à gauche*) et l'image en lumière polarisée (*à droite*) d'une coupe transversale d'un segment d'ostéone. Les lamelles osseuses opaques aux rayons X sont, en grande partie, éclairées en lumière polarisée. Toutefois, comme dans le quart inférieur gauche de la figure, il arrive que plusieurs stries anistotropes distinctes paraissent se confondre sur la radiographie en une seule bande épaisse opaque aux rayons X (× 600).

Fig. 2.—Côte humaine. Corrélation entre la microradiographie (*en haut*) et l'image en lumière polarisée (*en bas*) d'une coupe longitudinale d'un ostéone. Les bandes les plus calcifiées coïncident avec les bandes anisotropes (× 640).

Fig. 3.—Fémur humain. Corrélation entre la microradiographie (*en haut*) et l'image en lumière polarisée (*en bas*) d'une travée d'os spongieux. Biréfringence et calcification élevée vont de pair (× 640).

grâce à une méthode suggérée par Baud [1] : les négatifs photographiques sont imprimés par contact sur films minces qui sont alors superposés et examinés par transparence.

Des corrélations semblables ont été obtenues avec des coupes décalcifiées après déposition aux rayons X, mais, dans ce cas, les dimensions des diverses structures se modifient assez souvent.

Discussion.—Il n'est pas douteux que la striation révélée par la microradiographie se rapporte à des différences de calcification. Les zones qui apparaissent blanches sur les clichés aux rayons X sont plus minéralisées que celles qui apparaissent noires [3]. Les lamelles les plus calcifiées coïncident le plus souvent avec des lamelles biréfringentes, aussi bien en coupe transversale qu'en coupe longitudinale. Il arrive que plusieurs stries anisotropes distinctes se confondent sur la radiographie en une seule bande épaisse opaque aux rayons X (Fig. 1). Inversement, une légère biréfringence peut s'observer dans quelques zones peu calcifiées, si l'on fait tourner la préparation sur la platine du microscope. Cependant, dans la majorité des cas, anisotropie et calcification élevée en calcium semblent aller de pair.

On sait que le microscope électronique a introduit une distinction entre lamelles fibrillaires d'une part et lamelles cémentantes [6] ou arciformes [4] d'autre part. Selon Rouiller [5], les lamelles fibrillaires, anisotropes, contiendraient des fibres collagènes serrées disposées en treillis, tandis que les lamelles cémentantes, isotropes, seraient pauvres en collagène et riches en substance fondamentale et en sels minéraux.

Nos observations concordent mal avec ces données du microscope électronique. Avec la technique microradiographique, nous n'avons pas pu montrer que la calcification d'une lamelle osseuse est en raison inverse de sa densité fibrillaire.

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STUDIES ON NUCLEAR ACTIVITY IN THE DIFFERENTIATION OF THE SEA URCHIN EMBRYO[1]

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The present work constitutes part of a programme outlined by Professor J. Runnström [10, 11]. Its aim is to study the cytological basis of the sea urchin development under normal as well as under experimentally changed conditions.

Material and methods.—The work was performed during Spring 1956 at Stazione Zoologica, Naples, and the material used was mainly *Paracentrotus lividus*. The stages between the early blastula and the early pluteus will be considered. The material was fixed in Carnoy fluid (absolute alcohol–chloroform–glacial acetic acid 6:3:1) and in Serra fluid (80 per cent alcohol–formalin–glacial acetic acid 6:3:1). Deparaffinized sections were stained in methylgreen-pyronine of Unna as modified by Brachet [1], with the Feulgen technique [2], and with the prussian blue method of Hale as modified by Immers [5]. This latter technique involves an impregnation of the sections with ferric acetate. After washing, the ferric ions bound to acid groups are made visible by treatment of the section with potassium ferrocyanide. For digesting ribonucleic acid (RNA) a crystalline preparation of ribonuclease (RNAse) was used.

Vegetalization was induced by exposure to sea water containing lithium ions [4] and animalization by adding iodosobenzoic acid [12] or zinc ions [8] to sea water.

Observations.—In Carnoy-fixed gastrula stages stained with methylgreen-pyronine, the cytoplasm was definitely more stained with pyronine in the entoderm and the most vegetal region of the ectoderm than in other parts of the larvae. Also the nuclei stained heavily with pyronine as well as with methyl green, and it seemed that the

[1] The work was supported by the Swedish Natural Science Research Council and the Swedish Cancer Society.

nuclei of the animal part accumulated more of both stains than did the nuclei of other regions.

These differences with respect to staining properties arose at the stage of immigration of the primary mesenchyme cells. Vegetalized larvae showed a heavier and more uniform cytoplasmatic pyronine staining than animalized larvae, while the accumulation of stain in the nuclei was stronger in these latter larvae. The same results were obtained when working with material fixed according to Serra, and after digestion with RNAse there were never more than traces of a pyronine stain in any sections. No definite quantitative differences between the nuclei of different regions were obtained when the Feulgen technique was applied. Also the differences between animalized and vegetalized larvae were, if any, inconspicuous.

The Hale procedure was primarily used by Immers for demonstration of acid polysaccharides in sea urchin eggs. Besides, however, he observed [6] that the nuclei of larvae animalized by temporary exposure to sulphate-free sea water were heavily stained with the Hale reagent, whereas the nuclei of control larvae showed at the most a very weak staining with the same reagent. Incidentally the present author noticed a pronounced increase in the Hale staining of the nuclei of Carnoy-fixed, sectioned, and deparaffinized material after pretreatment with a 1/10-molar acetate buffer solution, pH 6.0, at 30°C for 30 minutes. With the possible exception of the region adjacent to the nucleus, there was no increase in the staining properties of the cytoplasm. In such buffer-treated sections, the nuclei of the animal region were strongly stained while the entoderm and mesenchyme nuclei appeared definitely weaker in colour. Prior to the immigration of the primary mesenchyme cells there was no such Hale "gradient". In the cleavage and blastula stages the resting nucleus was hardly stained, but after buffer treatment the chromosomes appeared very distinct at metaphase. As in the case of methylgreen-pyronine staining there was a pronounced difference between vegetalized and animalized larvae. Thus, the nuclei of lithium-treated larvae were pale while the nuclei of larvae treated with isobenzoic acid or zinc were heavily stained in all parts of the larvae. Also, without buffer treatment, the nuclei of animalized larvae showed a positive Hale reaction. The results obtained by Immers have thus a general validity, irrespective of the way in which animalization is brought about.

The nature of the buffer-effect in the Hale staining.—The optimum staining properties were obtained in the Hale procedure when the sections were treated with a 1/10-molar buffer solution, pH 7.5, at 40°C for about 30 minutes. Acetate or citrate buffer was used with no perceptible difference in the result. No staining occurred after fixation in mixtures containing formalin, or when the Carnoy-fixed and deparaffinized sections were subjected to formaldehyde before the buffer treatment. If RNAse was added to the buffer solution the nuclei were not stained at all by the Hale procedure. This might suggest that the ferric ions have reacted with RNA. Because of the thorough washing of the sections after buffer pretreatment the possibility of the buffer solution affecting the Hale reagent could be neglected. The buffer solution may cause a splitting process which is prevented by fixation in formalhyde, but further analysis has shown that more likely an enzyme is present which is activated by the buffer treatment: an enzyme affecting RNA and which moreover is inactivated by formaldehyde. In fact RNAse is such an enzyme and it may be inferred from recent investigations that it acts in such a way that phosphate

groups in RNA may become available for a reaction with the ferric ions in the Hale reagent.

Such activation of endogenous RNAse in deparaffinized sections has earlier been demonstrated in plant tissue [9]. Related problems are also discussed by Lagerstedt [7].

The above explanation of the buffer effect was further tested by immersing deparaffinized sections treated with formaldehyde in a buffer solution containing $\frac{1}{500}$ mg RNAse per ml ($\frac{1}{50}$ of the concentration previously used in this investigation). Then, the increased staining capacity returned and there was no difference as compared with the result of the primary buffer experiment. This shows, moreover, that the gradient with respect to Hale staining does not correspond to differences in RNAse activity along the egg axis.

It was also possible to obtain the same results in Serra-fixed larvae if the buffer contained traces of RNAse, and if the sections were previously extracted with ether-alcohol in order to substitute the phosphatide-removing effect of the Carnoy fluid.

Discussion.—There seem to be different types or states of RNA in the cell as reflected by the different results obtained with the Hale reagent and with the pyronine stain. While the Hale-positive RNA is bound to the nuclei and increases towards the animal pole, the cytoplasmatic RNA as demonstrated by staining with pyronine seems to accumulate somewhat more towards the vegetal pole. The differences appear at the time of immigration of the primary mesenchyme cells, and Gustafson has shown [3] that this is also the stage for an increase in the number of mitochondria as well as in the activity of a number of enzymes. There may be a causal relation between those observations [3] and the results presented in this investigation.

The studies on animalized larvae demonstrate that the region of positive Hale staining expands in these larvae, whereas in vegetalized larvae the Hale staining remains weak all over the larva. The opposite may be valid for the cytoplasmatic RNA but this question needs further investigation. The differences in nuclear RNA discussed in this paper may therefore reflect processes important for the animal-vegetal balance in the sea urchin embryo.

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